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Efflux pump activity in drug resistance of *Staphylococcus aureus*

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Thesis Publications

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Costa SS, Falcão C, Viveiros M, Machado D, Martins M, Melo-Cristino J, Amaral L, Couto I. Exploring the contribution of efflux on the resistance to fluoroquinolones in clinical isolates of *Staphylococcus aureus*. BMC Microbiol. 2011;11:e241.

Costa SS, Mourato C, Viveiros M, Melo-Cristino J, Amaral L, Couto I. Description of plasmid pSM52, harboring the gene for Smr efflux pump, and its involvement in resistance to biocides in a MRSA strain. Int J Antimicrob Agents. 2013;41:490-2.

Costa SS, Viveiros M, Amaral L, Couto I. Multidrug efflux pumps in *Staphylococcus aureus*: an update. Open Microbiol J. 2013;7:59-71.

Costa SS, Junqueira E, Palma C, Viveiros M, Melo-Cristino J, Amaral L, Couto I. Resistance to antimicrobials mediated by efflux pumps in *Staphylococcus aureus*. Antibiotics. 2013;2:83-99.

To my father.

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Resumo

Efflux pump activity in drug resistance of *Staphylococcus aureus*

Sofia Maria Mourão Marques dos Santos Costa

Palavras-Chave: *Staphylococcus aureus*; bombas de efluxo; fluoroquinolonas; biocidas; multirresistência

Staphylococcus aureus é um importante agente patogénico, para o qual estão descritos vários mecanismos de resistência a compostos antimicrobianos, sendo a resistência mediada por bombas de efluxo (MDR-EP) o menos bem caracterizado. Foi objectivo desta Dissertação avaliar a contribuição global destes sistemas de efluxo, em particular, de NorA, Smr e QacA, para a resistência a fluoroquinolonas e a outros compostos antimicrobianos em *S. aureus*.

Foi estudada uma colecção de isolados clínicos de *S. aureus* resistentes à ciprofloxacina e estirpes de referência. A actividade de efluxo foi avaliada por metodologias baseadas no transporte de brometo de etídeo (EtBr), substrato de MDR-EPs, e pela determinação da concentração mínima inibitória para substratos de MDR-EPs na presença/ausência de inibidores de efluxo. A informação obtida foi complementada pela análise por RT-qPCR da expressão de genes que codificam para as principais MDR-EPs de *S. aureus* e seus reguladores e pesquisa de mutações associadas aos principais fenótipos de resistência estudados.

A relevância de NorA para a resistência aos compostos antimicrobianos foi analisada através da resposta de *S. aureus* ao *stress* imposto pelo EtBr. Demonstrou-se que a presença/ausência deste composto promove o aumento/decréscimo da expressão do gene *norA* com consequente redução/aumento da susceptibilidade a fluoroquinolonas, biocidas e corantes. Foram também analisados os alelos de *norA* dos isolados clínicos, tendo sido verificada a predominância do alelo *norAI*. Estes estudos evidenciaram a complexidade da regulação deste sistema, com a identificação de múltiplos factores que contribuem para a modelação da expressão de *norA* e actividade de NorA.

Em relação às bombas codificadas em plasmídeos, verificou-se que tanto QacA, codificada num plasmídeo associado à multirresistência, como Smr, codificada num plasmídeo sem genes de resistência adicionais, desempenham um papel importante na resistência a biocidas, podendo contribuir para a persistência e disseminação em ambiente hospitalar de estirpes resistentes a biocidas e potencialmente resistentes a antibióticos.

A caracterização global dos isolados de *S. aureus* de origem clínica revelou uma contribuição significativa do efluxo para a resistência aos compostos antimicrobianos, com a identificação de um grupo de isolados com actividade de efluxo aumentada e correlacionável com susceptibilidade reduzida a fluoroquinolones e biocidas. A incubação com inibidores de efluxo, em particular as fenotiazinas, promoveu uma redução dos níveis de resistência, sem contudo resultar na reversão do fenótipo de resistência. Os estudos de expressão génica não revelaram uma correlação directa entre maior actividade de efluxo e expressão génica, sugerindo que os isolados clínicos podem já estar adaptados para uma resposta por efluxo na presença de compostos nocivos. Observou-se ainda uma multiplicidade de respostas mediadas por efluxo aos diferentes substratos e suas concentrações, variáveis no padrão temporal de expressão génica, níveis de expressão e genes envolvidos.

Os resultados obtidos evidenciam que o efluxo constitui parte da resposta inicial da célula aos compostos antimicrobianos, a qual, no caso das fluoroquinolonas, é seguida pela aquisição de mutações nos genes alvo. Mostrou-se ainda que a pressão exercida por biocidas induz a resistência cruzada a fluoroquinolonas.

Em resumo, os resultados descritos nesta Dissertação demonstram a contribuição do efluxo para a emergência de resistência a fluoroquinolonas e outros compostos antimicrobianos e o seu papel na emergência de estirpes de *S. aureus* multirresistentes em ambiente hospitalar.

Abstract

Efflux pump activity in drug resistance of *Staphylococcus aureus*

Sofia Maria Mourão Marques dos Santos Costa

Keywords: *Staphylococcus aureus*; efflux pumps; fluoroquinolones; biocides; multidrug resistance.

Staphylococcus aureus is a major human pathogen, for which several resistance mechanisms have been described. Of these, resistance mediated by multidrug efflux pumps (MDR-EP) is characterized to a lesser extent. The aim of this Dissertation was to evaluate the overall contribution of MDR-EP, in particular, of NorA, Smr and QacA to *S. aureus* resistance towards fluoroquinolones and other antimicrobials.

The collection studied comprised ciprofloxacin-resistant *S. aureus* clinical isolates plus reference strains was studied. Efflux activity was assessed by methodologies based on the MDR-EP substrate ethidium bromide (EtBr) and determination of minimum inhibitory concentrations of MDR-EP substrates in the presence/absence of efflux inhibitors. RT-qPCR was used to evaluate the expression level of MDR-EP genes and their regulators. Mutations associated with resistance were screened in genes of interest.

The relevance of NorA to antimicrobial resistance was analyzed in the *S. aureus* stress response to EtBr. It was shown that the presence/withdrawal of this compound promoted an increase/decrease of *norA* expression level and subsequent reduction/increase of susceptibility towards fluoroquinolones, biocides and dyes. We also analyzed the *norA* alleles within the clinical isolates collection and found that n*orAI* was the prevalent allele. The results obtained substantiated the intricate regulatory system of NorA, with the identification of multiple factors that contributed to the modulation of the *norA* gene and NorA activity.

The study of QacA, encoded on a multiresistance plasmid and Smr, encoded on a small plasmid with no additional resistance genes, revealed their important role on the resistance to biocides in the isolates that carry these determinants. Overall, the data indicates that these MDR-EPs and respective plasmids may contribute to the persistence

and proliferation of biocide-resistant strains and potentially antibiotic-resistant strains in the hospital environment.

The characterization of the clinical isolates collection revealed efflux as an important component of resistance, with detection of a group of isolates with increased efflux activity, which could be correlated with increased resistance to fluoroquinolones, biocides and dyes. The efflux inhibitors tested, in particular phenothiazines, were efficient in reducing resistance levels, yet without promoting reversion of the resistance phenotype. Gene expression assays revealed no direct correlation between increased efflux activity and levels of gene expression, suggesting that clinical isolates may be primed to efflux noxious compounds. A multiplicity of efflux-mediated responses to different inducers and their concentrations was observed, varying in the temporal pattern of expression, genes overexpressed and levels of expression.

The results obtained support the role of efflux as a first-line response of *S. aureus* to antimicrobial compounds, which in the case of fluoroquinolones, is followed by occurrence of target-based mutations. Evidence was also obtained showing that the pressure exerted by biocides can lead to cross-resistance to fluoroquinolones.

In summary, the results described in this Dissertation demonstrate that efflux contributes to resistance towards fluoroquinolones and biocides and plays a key role on the emergence of *S. aureus* multiresistant strains in the healthcare environment.

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List of abbreviations

A – adenine
ABC – adenosine 5'-triphosphate (ATP)-binding cassette
ACR – acriflavine
Ala – alanine
BAC – benzalkonium chloride
BER – berberine
C – citosine
CA-MRSA – community-associated methicillin-resistant *Staphylococcus aureus*
CCCP – carbonyl cyanide *m*-chlorophenylhydrazone
cDNA – complementary DNA
CET - cetrimide
CHL – chloramphenicol
CHX – chlorhexidine diacetate
CHXg – chlorhexidine digluconate
CIP – ciprofloxacin
CoNS – coagulase-negative staphylococci
CPZ – chlorpromazine
C_T – threshold cycle
CTAB – hexadecyltrimethylammonium bromide
CV- crystal violet
D/Asp – aspartic acid
Del – deletion
DMSO – dimetilsulphoxide
DNA – deoxyribonucleic acid
dNTP – deoxyribonucleoside triphosphate
DQ – dequalinium chloride
E/Glu – glutamic acid
ECOFF – epidemiological cut-off value
EI - efflux inhibitor
ERY – erythromycin
EtBr – ethidium bromide
F/Phe – phenylalanine
G – guanine
G/Gly – glycine

GEN – gentamycin
HA-MRSA – hospital-associated methicillin-resistant *Staphylococcus aureus*
Ins – insertion
IS – insertion sequence
K/Lys – lysine
K - guanine / timine.
Lac – lactate
LA-MRSA – livestock-associated methicillin-resistant *Staphylococcus aureus*
LEV – levofloxacin
M - adenine / citosine
MATE – multidrug and toxic compound extrusion
MBC – minimum bactericidal concentration
MDR – multidrug resistance
MFS – major facilitator superfamily
MGE – mobile genetic element
MHB – Mueller-Hinton broth
MIC – minimum inhibitory concentration
MLST – multilocus sequence typing
MRSA – methicillin-resistant *Staphylococcus aureus*
MSSA – methicillin-susceptible *Staphylococcus aureus*
N/Asn – asparagine
NAL – nalidixic acid
NOR – norfloxacin
OD – optical density
ORF – open reading frame
OXA – oxacillin
P/Pro - proline
PBP – penicillin-binding protein
PBS – phosphate buffered saline
PCR – polymerase chain reaction
PEN – penicillin
PFGE – pulsed-field gel electrophoresis
PMF – proton motive force
PT – pentamidine isothionate salt
Q/Gln – glutamine
QAC – quaternary ammonium compounds
RACE-PCR – rapid amplification of cDNAs ends PCR

RES – reserpine
RIF – rifampicin
RNA – ribonucleic acid
RND – resistance-nodulation-cell division
RT – room temperature
RT-qPCR – quantitative reverse transcription polymerase chain reaction
S/Ser – serine
SCC*mec* – staphylococcal cassette chromosome *mec*
SMR – small multidrug resistance
SPX – sparfloxacin
ST – sequence type
T – timine
TET – tetracycline
TMS – transmembrane segment
Tn – transposon
TPP – tetraphenylphosphonium bromide
TSA – tryptone soy agar
TSB – tryptone soy broth
TZ – thioridazine
UTR – untranslated region
VER – verapamil
VISA – vancomycin-intermediate resistant *Staphylococcus aureus*
VRSA - vancomycin-resistant resistant *Staphylococcus aureus*
W - adenine / timine
Y/Tyr – tyrosine

List of Units

bp – base pair; kb – kilobase; Mb – megabase
ºC – degrees Celsius
rpm – rotation per minute
msec – millisecond; sec – second;
min – minute; h – hour
nm – nanometer; µm – micrometer;
cm – centimeter
mL – milliliter; L – liter
µg – microgramа; mg – milligram
pmol – picomole
mM – millimolar; M - molar
cfu – colony forming unit
µF – microfaraday
kV – kilovolt
Ω - ohm

CHAPTER 1

General Introduction

1. General Introduction

1.1. *Staphylococcus aureus*: the making of a major pathogen

The species *Staphylococcus aureus* is one of the 48 species comprised in the *Staphylococcus* genus, which was established in 1883 to describe a group of bacteria responsible for inflammation and suppuration [48, 100]. During the 20th century, the classification criteria of this genus underwent several revisions [100]. Currently, it includes Gram-positive cocci with a diameter varying from 0.5 to 1.5 μm that can occur as single cells, in pairs, tetrads, short chains or as irregular grape-like clusters. These cocci are non-motile, non-sporogenic, and in some cases possessing a microcapsule. They are facultative anaerobes and producers of the enzyme catalase, with the exception of *Staphylococcus aureus* subspecies *anaerobius*, *Staphylococcus saccharolyticus* and some strains of *Staphylococcus aureus* subsp. *aureus*. *S. aureus* (Figure 1.1) is a producer of the enzyme coagulase, a trait traditionally used to differentiate it from other species, generally designated as coagulase-negative staphylococci (CoNS). Nonetheless, other species are also coagulase-positive, including *Staphylococcus intermedius*, *Staphylococcus pseudintermedius*, *Staphylococcus delphini* and some strains of *Staphylococcus hyicus*, among others [32, 100].

Figure 1.1. Image of a *S. aureus* strain grown in blood-agar medium.

Staphylococci are ubiquitous in nature, although their presence is more common in the skin, skin glands and mucous membranes of mammals, in a permanent or transient status [32, 100]. Primates are the natural hosts of *S. aureus*, although they can also be found in domestic animals, poultry, hares and rodents [32].

In humans, the anterior nares are a preferable niche of *S. aureus*. Several studies have revealed different patterns of *S. aureus* nasal carriage in the human population, ranging from persistent to intermittent [101, 223]. Overall, among the general adult population the mean carriage rate is approximately 30 percent [223]. Nasal carriage can be influenced by several factors, such as age, race and the immunological status of the

individuals [101, 223]. It is recognized as a key factor for the development of *S. aureus* infections, with the anterior nares being proposed as the potential starting point of propagation of *S. aureus* strains to other sites of the body like the skin. It has been also shown that persistent carriers have higher *S. aureus* loads and an elevated risk for acquisition of *S. aureus* infections [101, 223]. Although the majority of *S. aureus* infections are attributable to colonizing strains, transmission can also occur, either directly, by skin-to-skin contact, or indirectly by contact with contaminated surfaces and objects [123].

Infections caused by this bacterium can take the form of mild superficial lesions, such as the skin and soft-tissue infections cellulitis and impetigo, more common in community-associated infections; life-threatening systemic infections like bacteremia, endocarditis, pneumonia and osteomyelitis, more common in nosocomial-associated infections; and toxin-mediated syndromes, such as food poisoning, scalded skin syndrome and toxic shock syndrome [32, 123]. *S. aureus* is a frequent primary pathogen [139]. In general, for an infection to take place, a first step of local colonization of the tissue surface occurs, followed by an invasion of the local tissue, an evasion from the host defense mechanisms and a final dissemination to other body sites [123]. *S. aureus* elaborates a plethora of virulence factors that play an important role in the pathogenesis of the infection [32]. These virulence factors are involved in attachment (e.g., collagenase and clumping factors), in tissue invasion (e.g., proteases, lipases and hyaluronidase) and in evasion of the host defense mechanisms (e.g., leukocidins, like Panton-valentine leukocidin and γ-toxin, and protein A) [32, 43, 62]. Some strains can also secret additional virulence factors involved in toxin-mediated syndromes, such as the toxic shock syndrome toxin-1 (TSST-1), several enterotoxins (SEA, SEB, SECn, SED, SEE, SEG, SHE, and SEI) responsible for food poisoning and exfoliative toxins (ETA and ETB), responsible for the scalded skin syndrome [32, 43, 62]. Other virulence factors, such as coagulase, the arginine catabolic mobile element and bacteriocin have a poorly defined role in virulence and pathogenesis [62]. The *S. aureus* capacity to produce this array of virulence factors contrasts with the less virulent CoNS, some of which can cause opportunistic infections in humans.

Staphylococci present a low G + C content that varies between 30 and 38%. To date, 46 complete genome sequences of *S. aureus*, ranging in size from 2.72 Mb to 3.08

Mb, have been made available (http://www.ncbi.nlm.nih.gov/genome). Comparative analysis of the earlier published genomes revealed that 75% of the genome, named "core" genome, is conserved and mainly comprised of genes with housekeeping functions, whereas the remaining 25%, the "accessory" genome, is variable and constituted by mobile genetic elements (MGEs) [117]. These MGEs include integrated prophages, genomic islands, pathogenecity islands, staphylococcal cassette chromosomes (SCC), plasmids, transposons (Tn) and insertion sequences (IS), playing an important role in the pathogenesis, virulence, resistance and host-adaptation of *S. aureus* given that they often carry virulence, toxin and resistance determinants [60, 130, 134].

Besides its pathogenic potential and virulence, *S. aureus* also shows a remarkable ability to develop and/or acquire a diversity of resistance mechanisms towards antibiotics and other antimicrobial compounds. Of major concern are the methicillin-resistant *S. aureus* (MRSA) strains, resistant to all β-lactam antibiotics. Since the description of the first MRSA strain, more than five decades ago, they have become a major cause of nosocomial infections worldwide [23, 40, 145]. These MRSA strains with onset in hospitals are denominated hospital-associated MRSA (HA-MRSA). MRSA infections are associated with an increased burden, adding to infections caused by methicillin-susceptible *S. aureus* (MSSA) [63, 102]. The association between MRSA bacteremia and higher mortality rates is still debatable [63, 102, 217]. However, there is strong evidence that MRSA infections are implicated in a higher morbidity [63] and higher hospital costs [63, 119]. More recently, MRSA strains with onset in the community have emerged, being designated community-associated MRSA (CA-MRSA). These strains were shown to be distinct from the traditional HA-MRSA strains; they belong to different clonal lineages, show diverse genetic backgrounds, and albeit more susceptible to antibiotics other than β-lactams, they are generally more virulent and transmissable, with less fitness burden, being mainly associated with skin and soft tissue infections although also potentially lethal [35, 40, 138]. Currently, the distinction between CA-MRSA and HA-MRSA strains is becoming less clear, as a result of the invasion of healthcare settings by CA-MRSA strains, especially in countries where its prevalence rate is higher. The eventual displacement of traditional HA-MRSA strains by CA-MRSA in healthcare settings has been also foreseen [36, 138, 145, 200] and

recent studies have reported that these CA-MRSA strains have acquired additional resistance determinants [138, 200]. More recently, new MRSA clones that are not related to contemporary CA-MRSA or HA-MRSA clones have emerged among livestock, being designated livestock-associated MRSA (LA-MRSA) [51]. Among these, a particular clone (ST398) was first detected in pig farmers and later found to be prevalent among pigs and in other food-producing animals like cattle and poultry [51]. Reports of the occurrence of this LA-MRSA clone in livestock have arisen from several countries, including cases of animal-to-person transmission [49]. This transmission was found to occur mainly in people in close contact with animals, like pig farmers and slaughterhouse workers, but these strains were found to be poor colonizers of humans and it was proposed that their carriage results from continued exposure rather than stable colonization [65]. Furthermore, they appear not to be associated with a high infectious risk for humans [51]. A recent study revealed a likely human ancestral origin for this clonal lineage as MSSA that experienced a human-to-livestock jump followed by host adaptation to livestock and acquisition of additional resistance determinants [172]. Of particular interest is the identification of new resistance determinants in these LA-MRSA strains [98, 221]. These findings highlight the potential role of *S. aureus* as a zoonotic pathogen and of animals as reservoirs for new MRSA strains [72, 172]. These LA-MRSA strains may also act as a reservoir for the emergence and dissemination of new resistance genes. Similarly, reports on the occurrence of MRSA strains in a variety of food samples like meat products, milk, fish and other food products have been published, but information is still scarce to support the role of MRSA as a potential food-borne pathogen [5, 222].

Epidemiological surveillance programs have shown that only a few MRSA epidemic clones are responsible for the majority of the nosocomial infections worldwide and that the prevalence rates vary considerably amongst different countries [40, 71, 200]. In Europe, MRSA strains are the main contributor to nosocomial infections [45], although the proportion of invasive MRSA isolates differs significantly amongst European countries in a north-to-south gradient, ranging from 0.3% in Norway to 54.6% in Portugal (Figure 1.2) [46].

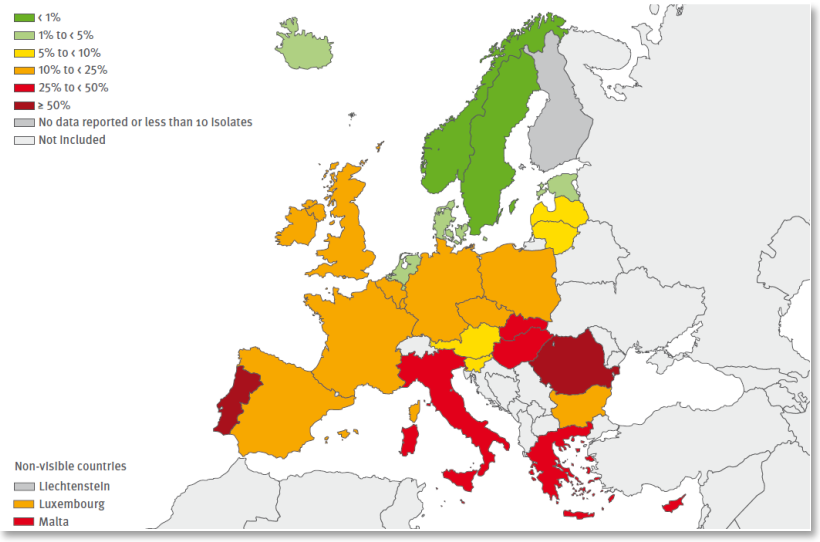


Figure 1.2. Map depicting the percentage (%) of invasive MRSA isolates reported in 2011 by 28 of the European countries participating in the European Antimicrobial Resistance Surveillance Network (EARS-Net). Reproduced from [46].

Some European countries have been indicating a decreasing trend of invasive MRSA infections [46], probably supported by application of improved measures of control and prevention of MRSA in healthcare settings [102]. Yet, at least one third of the European countries remain with proportion rates above 25% [46, 102]. In addition, some evidence indicates that this decrease is not followed by the overall rates of *S. aureus* infections, thus implying a rise in the number of MSSA infections [63].

Therefore, delineating the risk factors for acquisition of MSSA/MRSA infections, implementation of measures to control and prevent MRSA dissemination, such as: hand hygiene; MRSA carrier screening policies; environmental decontamination: patient isolation and decolonization; antibiotic stewardship and identification of reservoirs are of paramount importance; and are expected to promote a significant reduction in the rate of MSSA/MRSA infections [84, 113, 202]. Also, the continuous research on the epidemiology of *S. aureus* strains and on the emergence, acquisition and transmission of mechanisms of resistance to antibiotics and other antimicrobial agents is essential to better understand and manage this pathogen.

1.2. Resistance to antimicrobial agents in *S. aureus*

1.2.1. Antibiotics: modes of action and mechanisms of resistance

Antibiotics are natural, semi-synthetic or synthetic compounds with selective toxicity towards bacteria, causing death or growth inhibition, thus allowing the host defense mechanisms to cope with the infection [28]. Antibiotics can exert their antibacterial action by (Figure 1.3-A); (i) the inhibition of nucleotide biosynthesis (e.g., sulfonamides); (ii) the inhibition of DNA synthesis (e.g., quinolones); (iii) the inhibition of RNA synthesis (e.g., rifamycin); (iv) the inhibition of protein synthesis (e.g., macrolides); (v) the inhibition of cell wall biosynthesis (e.g., β-lactams); and (vi) the disorganization of the cell membrane (e.g., peptides) [28].

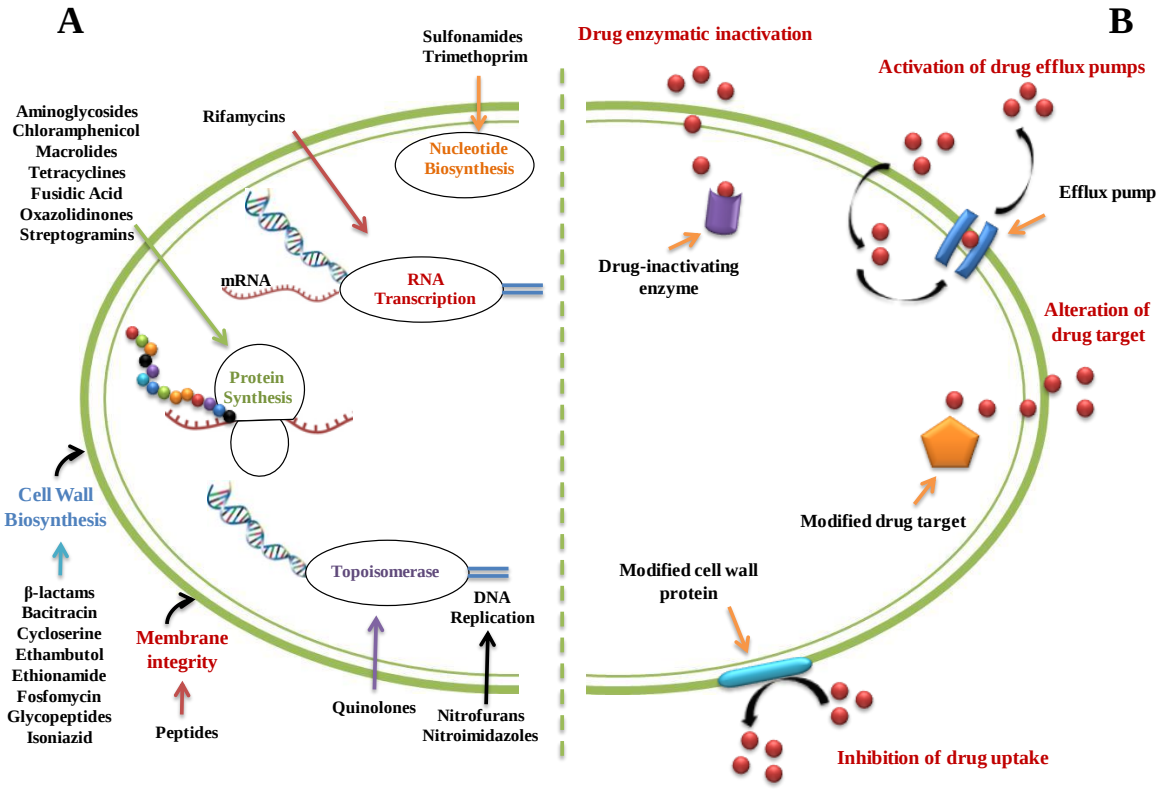


Figure 1.3. Representation of the modes of action of antibiotics in the bacterial cell (A) and the main mechanisms of resistance to surpass their activity (B).

The introduction of antibiotics to treat infectious diseases was one of the hallmarks of 20th century medicine. Shortly after, the first antibiotic-resistant bacteria were described. Since then, the development of new antibiotics has been accompanied by an increase in antibiotic-resistant bacterial strains and diversity of the mechanisms used to surpass their effect. These mechanisms include (Figure 1.3-B); (i) enzymatic degradation or modification of the antibiotic, exemplified by the action of β-lactamases that cleave the β-lactam ring of β-lactam antibiotics and acetylation of aminoglycosides by acetyltransferases, respectively [228]; (ii) modification of the target, such as the occurrence of mutations in the DNA gyrase and topoisomerase IV that diminishes their affinity to quinolones [112]; (iii) reduction of the intracellular concentration of the antibiotic, via its extrusion by efflux pumps and/or reduction of its entry by altered cellular permeability [108], (iv) protection of the target site, exemplified by the overproduction of peptidoglycan in *S. aureus* increasing the number of vancomycin target sites, thus trapping the antibiotic in the cell wall and preventing its action [122]. Nowadays, at least one mechanism of resistance is described for each class of commonly used antibiotics [34]. Specific physiological states can also influence the susceptibility of bacteria to antibiotics, such as biofilm formation, growth conditions that induce slow growth rates, as well as phenotypic variation within a bacterial population [78].

Some bacteria are intrinsically resistant to one or more classes of antibiotics, such as *Pseudomonas aeruginosa*, while others are naturally susceptible to almost all antibiotics, as in the case of *S. aureus*. Yet, bacteria that are initially susceptible to an antibiotic can develop and/or acquire resistance through spontaneous mutations in target genes or by the acquisition of exogenous genes [34]. These exogeneous genes are usually localized in MGEs, such as plasmids and transposons, and can be acquired by horizontal gene transfer, through transformation, conjugation or transduction processes [34]. Many bacterial species may show multidrug resistance (MDR) phenotypes, such as *S. aureus*, vancomycin-resistant enterococci, *Mycobacterium tuberculosis*, *Escherichia coli*, *Acinetobacter baumanii*, *Klebsiella* spp., *Enterobacter* spp., and *P. aeruginosa*, which may be responsible for major outbreaks in the hospital and/or in the community [225, 226].

1.2.2. Evolution of antibiotic resistance in *S. aureus*

In the pre-antibiotic era, the mortality rate associated with invasive infections of *S. aureus* surpassed 80% [198]. In the early 1940s, the β-lactam antibiotic penicillin was introduced in the clinical practice, registering a drastic reduction in the mortality rate associated with these infections. Nevertheless, penicillin-resistant strains emerged shortly after, first in hospitals and then in the community. Underlying this resistance was the acquisition of a plasmid-located gene encoding a penicillinase (β-lactamase), an enzyme that degrades the β-lactam ring rendering the antibiotic inactive [122]. By 1948, more than 50% of the *S. aureus* clinical isolates were β-lactamase producers, a number that increased significantly in the following years, currently reaching 80 to 90% of the total clinical isolates worldwide [40].

Following penicillin, new antibiotics were introduced in the clinical practice, including aminoglycosides, new β-lactams, chloramphenicol, tetracycline and macrolides. However, strains resistant to these agents emerged rapidly, as well as the first strains showing resistance to multiple antibiotics. Many of the resistance mechanisms to these antibiotics were encoded in MGEs, including large multiresistance plasmids [126].

To overcome resistance to penicillin, semi-synthetic β-lactam antibiotics were developed that were not susceptible to the action of β-lactamases, such as methicillin and oxacillin. However, resistance once again emerged swiftly and in the year following the introduction of methicillin in clinical practice were published the first reports of resistance in clinical strains [6, 87]. These MRSA strains emerged in the UK, but soon spread, first, to healthcare institutions in other European countries and, later on, around the world. The underlying mechanism of resistance to methicillin is the synthesis of an additional altered penicillin-binding-protein (PBP), PBP2a or PBP2', that shows a diminished affinity for β-lactam antibiotics [73, 216]. This additional PBP is coded by the *mecA* gene, comprised in the mobile element staphylococcal cassette chromosome *mec* (SCC*mec*) that integrates into the *S. aureus* chromosome at a specific site within *orfX* gene that encodes a ribosomal methyltransferase [15, 35]. To date, eleven SCC*mec* types and several subtypes have been indentified, with some carrying additional

resistance determinants to other antibiotics, such as aminoglycosides, macrolides, lincosamides and streptogramins as well as to heavy-metals like mercury [40, 194]. Recently, a new SCC*mec* element was described carrying a divergent *mec* gene [194].

Fluoroquinolones were developed to treat Gram-negative infections, showing a narrow spectrum of activity against Gram-positive bacteria [80]. Although not generally used to treat *S. aureus* infections, fluoroquinolone resistance emerged rapidly in this bacterium, probably due to an extensive exposure in the hospital environment. Resistance originates through spontaneous mutations in the two targets, DNA gyrase and topoisomerase IV and by efflux [79]. An interesting aspect of fluoroquinolone resistance in *S. aureus* is the build up of evidence as a selective factor for MRSA strains [80].

The introduction of glycopeptides, in particular vancomycin, was of major importance as they became key antibiotics in therapy against MRSA infections [64]. However, in 1997, the first case of a MRSA strain with reduced susceptibility to vancomycin was reported [77]. Since then, several cases have been described of strains with reduced susceptibility or intermediate resistance to vancomycin (VISA) in many countries [81]. The mechanism of reduced susceptibility to vancomycin was described as an alteration in the biosynthesis of peptidoglycan that results in the thickening of the cell wall and in the increase of available targets for vancomycin, the dipeptide D-Ala-D-Ala. The antibiotic is then sequestered in the thickened cell wall and its activity impaired [81, 195, 196]. In 2002, MRSA strains resistant to vancomycin (VRSA) were reported for the first time [19, 20]. These strains showed a different mechanism of resistance to vancomycin, namely the acquisition of the *vanA* operon, probably through conjugation with a strain of *Enterococcus faecalis*, which allows the synthesis of a precursor of the cell wall with an alternate terminal, D-Ala-D-Lac. This new dipeptide has a lower affinity for vancomycin thus allowing the bacteria to survive [220]. Since then, few cases of VRSA strains were reported, but the emergence of such strains alarmed both the medical and scientific community. The surfacing of VISA and VRSA strains made therapy of MRSA infections even more difficult [56, 64]. New antibiotics have been developed and may be used as a last resort for these serious infections although in many cases with no gain in efficacy. The most relevant of these antibacterial agents are the lipopeptide daptomycin and the oxazolidinone linezolide [56, 64]. Among

other therapeutical alternatives are the glycylcycline tigecycline, the lipoglycopeptide telavancin and the cephalosporin ceftaroline. However, resistance to these agents has already been reported [56, 64], including cross-resistance between vancomycin and daptomycin [33]. The use of older antibiotics, alone or in combination, is also being pondered as an option for standard therapy, including quinupristin-dalfopristin, trimethoprim-sulphamethoxazole, chloramphenicol and tetracycline [56, 64].

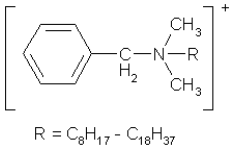
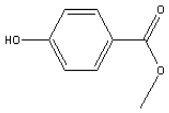
1.2.3. Biocides: modes of action and mechanisms of resistance

Biocides are compounds that possess a broad spectrum of action and inhibit cell growth or promote cell death [135]. They are classified according to their applicability; as antiseptics, when for application on living tissues, such as the skin and mucuous membranes; as disinfectants, for the decontamination of inanimate surfaces; as preservatives, for use in consumer products [59, 135]. Their use is widespread in healthcare settings, playing an important role in the prevention and control of nosocomial infections. Historically, biocides have been in use for several centuries, as exemplified by vinegar for the cleansing of wounds. However, it was in the late 19th century and early 20th century that other agents were introduced, including chlorine-releasing agents, phenols, organomercurials, cationic compounds, such as the quaternary ammonium compounds (QACs), and chlorhexidine [179]. A large assortment of molecules is currently registered as biocides in the USA and Europe [191]. The last decades have also witnessed a massive increase in the use of these compounds in consumer products, animal husbandry and in several industries, intensifying the exposure of the general population to these agents [59].

Biocides act distinctly from antibiotics; while antibiotics are effective at low concentrations upon a given cellular target, biocides are used at high concentrations and act upon multiple targets [59, 135]. However, for some biocides like triclosan, lower concentrations may allow an effect on a more specific target [135]. Biocides can be divided according to their mechanism of action (Table 1.1) as oxidants (e.g., halogens and peroxydes), which oxidize organic matter via radical-mediated reactions; as electrophiles (e.g., formaldehyde, isothyazolones), that inactivate enzymes by covalent

binding to cellular nucleophiles; as lytic (e.g., chlorhexidine and QACs), when they promote the destabilization of the cell membrane; or as protonophores (e.g., weak acids, parabens), which disturb the membrane pH gradient [24, 61, 146]. The activity of a biocide can be influenced by several factors, including its concentration, pH, temperature, the time of contact, formulation, presence of interfering material like organic matter, as well as the nature, load, localization and condition of the microorganisms upon which the biocide acts [178-181]. Biocides usually show low selectivity, acting against different types of microorganisms, with varying degree of activity against bacteria, spores, fungi, viruses and protozoa [181].

Table 1.1. Examples of commonly used biocides, their modes of action and applications.

Mode of action	Biocide class	Example	Use
Oxidizing	Halogens Peroxydes	Hydrogen peroxyde	Disinfectants Antiseptics
Electrophilic	Aldehydes Isothiazolones	 Glutaraldehyde	Disinfectants, Preservatives
Lytic	QACs Chorhexidine	 QACs	Disifectants Antiseptics
Protonophores	Parabens Weak acids	 Methylparaben	Preservatives

QAC: quaternary ammonium compound.

The extensive and widespread use of biocides has prompted concern about the possible emergence of biocide-resistant strains, and most importantly on the potential role of biocides as selective pressure for the emergence of antibiotic-resistant bacteria [21, 59, 129, 135, 140, 146, 180, 191, 224]. The multiple documented outbreaks associated with contaminated antiseptics and disinfectants strengthen these concerns [44, 219]. For these reasons, the European Union has adopted directives for the marketing of biocides [47] and Scientific Committees have been designated to evaluate the cumulative impacts and risks resulting from the use of biocides on antimicrobial

resistance [191, 192]. These questions are accompanied by a difficulty in establishing cut-off values to distinguish between biocide-resistant and biocide-susceptible bacteria, with many authors preferring to apply the expressions reduced susceptibility or tolerance instead of resistance. In the context of an antibiotic, when measuring minimum inhibitory concentration (MIC) values to establish the susceptibility of a bacterium to a given antibiotic, this cut-off value is expressed as the breakpoint, specific for each combination of antibiotic and bacteria and above which bacterial strains are deemed resistant to that antibiotic, consequently treatment failure is anticipated [121]. In the context of biocides, not used therapeutically, many of the parameters establishing breakpoints are not applicable. Furthermore, biocides are usually used in formulations, at high-concentrations, with one or more active ingredients and excipients, which enhance their activity. They have an unspecific effect on the cell and are required to achieve a rapid killing of bacteria. Thus, the use of MIC values to reflect changes in the susceptibility of bacteria to biocides is considered not the most appropriate methodology and no guidelines have been established to differentiate resistance from susceptibility. However, many studies use MICs since these may be useful for a first assessment of the expected effect of a biocide on a given microorganism [18, 129, 180]. In the current year, tentative biocide epidemiological cut-off (ECOFF) values, based on MIC and minimum bactericidal concentrations (MBC), have been proposed for some bacterial species, including *S. aureus*, and a restricted number of biocides, namely benzalkonium chloride, chlorhexidine, triclosan and sodium hypochlorite [9].

The susceptibility of bacteria to biocides is variable, with bacterial spores being the less susceptible, followed by mycobacteria and Gram-negative bacteria, while Gram-positive bacteria are the most susceptible to the action of these compounds [181]. The intrinsic tolerance found in mycobacteria and Gram-negative bacteria is closely related to the reduced permeability of the cell wall to these compounds [39, 111]. Gram-positive bacteria, in particular *S. aureus*, possess a permeable cell wall which does not oppose to the entry of biocides and many antibiotics, rendering the bacteria susceptible to these compounds [111]. As mentioned, biocides act on multiple cell targets, thus emergence of resistance through acquisition of mutations is regarded as unlikely. Nevertheless, reduced susceptibility to the bisphenol triclosan, which is commonly used in antiseptic wash preparations and recommended for MRSA decolonization [30], has

been associated with the occurrence of mutations in the gene *fabI* encoding an enzyme involved in fatty acid biosynthesis in several bacteria, including *S. aureus* [16], *E. coli* and *M. tuberculosis* (homolog *inhA*) [224].

In general, reduced susceptibility to biocides has been associated with chromosomally or plasmid-encoded efflux pumps that are capable of extruding a broad range of substances [168, 170]. For example, it has been shown that exposure to triclosan and household antimicrobial cleaning agents promoted an efflux-mediated reduced susceptibility to those biocides and several antibiotics, such as tetracycline, ciprofloxacin and trimethoprim in Gram-negative bacteria [29, 205]. Resistance to biocides may also emerge by acquisition of MGEs. A wide variety of plasmids have been identified carrying resistance determinants to cationic compounds and heavy-metals [168, 170, 177]. In the last two decades, plasmid-borne resistance to several biocides, including chlorhexidine, cetrimide, triclosan and benzalkonium chloride has been reported in both Gram-negative and Gram-positive bacteria [59, 168, 170].

1.2.4. Resistance to biocides in *S. aureus*

As a prevalent nosocomial pathogen, *S. aureus* is under constant exposure to antiseptics and disinfectants. Among standard actions for prevention of nosocomial infections, hand hygiene is of paramount importance. Hand formulations are mainly alcohol-based (ethanol and/or isopropanol), many times supplemented with other biocides, such as chlorhexidine, QACs, triclosan and povidone-iodine [227]. Adding up to these measures, guidelines for MRSA infection control generally rely on the use of biocides for the skin decolonization of patients [30].

As aforementioned, *S. aureus* is naturally susceptible to most biocides. Yet, intrinsic tolerance to biocides may occur in VISA strains due to the thickening of the cell wall [111], although no specific data on this has been provided so far. Resistance to biocides in *S. aureus* has been mainly associated with the presence of plasmid-borne efflux systems, namely QacA/B and Smr. Their encoding genes are found in clinical, animal and food isolates of *S. aureus* as well as in other staphylococci [170]. These and other efflux pumps, namely QacG, QacH and QacJ, first identified in food and animal

isolates [14], and more recently in human clinical isolates [232], have the capacity to expel several biocides, like QACs, chlorhexidine, diamidines and dyes and have been correlated with reduced susceptibility towards those compounds [14, 170]. Of particular interest is the occurrence of these genes on multiresistance plasmids of *S. aureus* and other staphylococci that already convey resistance to several antibiotics and heavy-metals. In addition, all chromosomally multidrug resistance (MDR) efflux pumps described so far in *S. aureus*, including NorA, NorB, NorC, MepA and MdeA have the potential to extrude biocides. However, the assessment of their involvement in biocide resistance is more difficult to ascertain, since these chromosomal efflux pumps occur naturally and the evaluation of the role played by each individual pump is a complex task. Nevertheless, some studies have associated a biocide reduced susceptibility phenotype to the overexpression of the genes coding for these pumps [31, 83, 105].

1.3. Multidrug efflux pumps

Efflux pumps are present in both eukaryotic and prokaryotic cells. The analysis of several published genomes reveals that drug efflux pumps may constitute 6% to 18% of all the transporters found in any bacterial cell [108]. The physiological role of these systems in bacteria has been related to the elimination of endogenous metabolites that are noxious to the cell, the secretion of virulence determinants, and in cell stress responses, suggesting that antimicrobial compounds are fortuitous substrates of these transporters [167, 171].

The first efflux systems to be associated with antibiotic resistance were identified in 1980 in tetracycline-resistant isolates of *E. coli* and corresponded to Tet proteins [137]. Since then, numerous efflux systems have been identified either in the chromosome or in plasmids of bacteria and associated with resistance to antibiotics and other antimicrobial agents [168, 170].

Efflux pumps can be classified according to their energy source, substrate specificity or filogenetic relations [163]. Bacterial efflux systems can be either specific, extruding only one antibiotic or class of antibiotics, or capable of extruding several

classes of antimicrobial compounds, being designated MDR efflux pumps. Concerning the energy source, efflux systems are divided in primary active transporters, which use the free energy of ATP hydrolysis to extrude compounds against a concentration gradient, exemplified by ABC transporters [103] and secondary active transporters that use the electrochemical potential gradient of the cytoplasmic membrane, the proton motive force (PMF), to drive the extrusion of their substrates by an antiport drug:H^+ mechanism. This type of transporter includes pumps of the major facilitator superfamily (MFS) [52, 159, 182], the small multidrug resistance (SMR) family [7, 8], the multidrug and toxic compound extrusion (MATE) family [110] and the resistance-nodulation-cell division (RND) superfamily [154] (Figure 1.4). Some MATE transporters can also use the membrane gradient of sodium ions as energy source [110].

Gram-negative and Gram-positive bacteria differ in the organization and composition of their cell wall. Whilst Gram-negative bacteria possess two membrane layers spaced by the periplasm, Gram-positive bacteria have a single cell membrane. Thus, the majority of the efflux systems present in Gram-negative bacteria is a tripartite complex that may belong to the RND, ABC or MFS families (Figure 1.4) [173]. In Gram-positive bacteria, the efflux systems are composed of a single component that traverses the entire cell membrane and belong to the MFS, MATE, SMR or ABC families (Figure 1.4) [173].

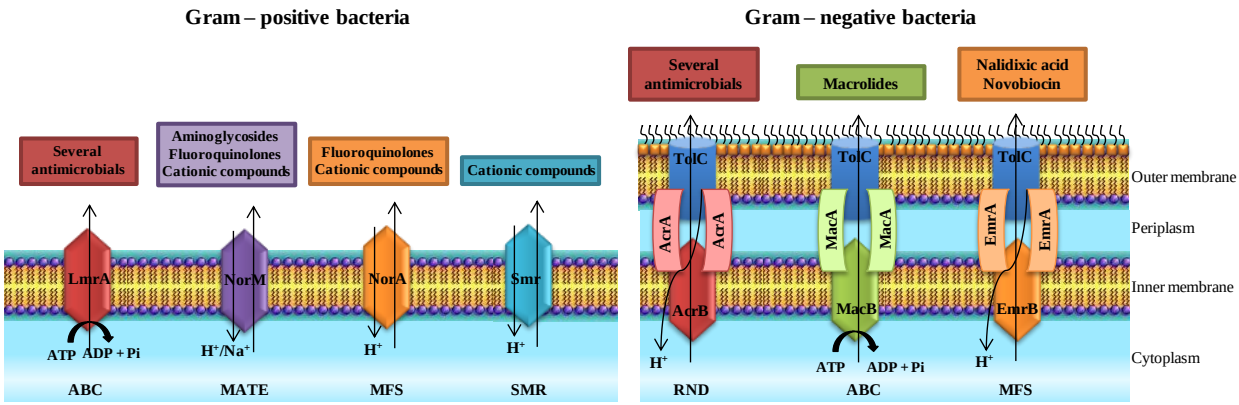


Figure 1.4. Representation of the five families of MDR efflux pumps and their distribution among Gram-positive and Gram-negative bacteria.

1.3.1. Major Facilitator Superfamily (MFS)

The Major Facilitator Superfamily comprehends more than 1,000 transporters grouped in, at least, 59 families among prokaryotic and eukaryotic cells [52, 182]. They are associated with the transport via antiport or sinport of several molecules, including antimicrobials, sugars, intermediates of the Krebs cycle and oligosaccharides [159, 182]. Hydropathy analysis of the MFS members supported a further division of the transporters according to their number of transmembrane segments (TMS), either 12 or 14 (Figure 1.5) [163]. Representatives of this superfamily are the *S. aureus* efflux pumps QacA/B and NorA, *E. coli* YdiY and EmrB and *Mycobacterium smegmatis* LfrA [182].

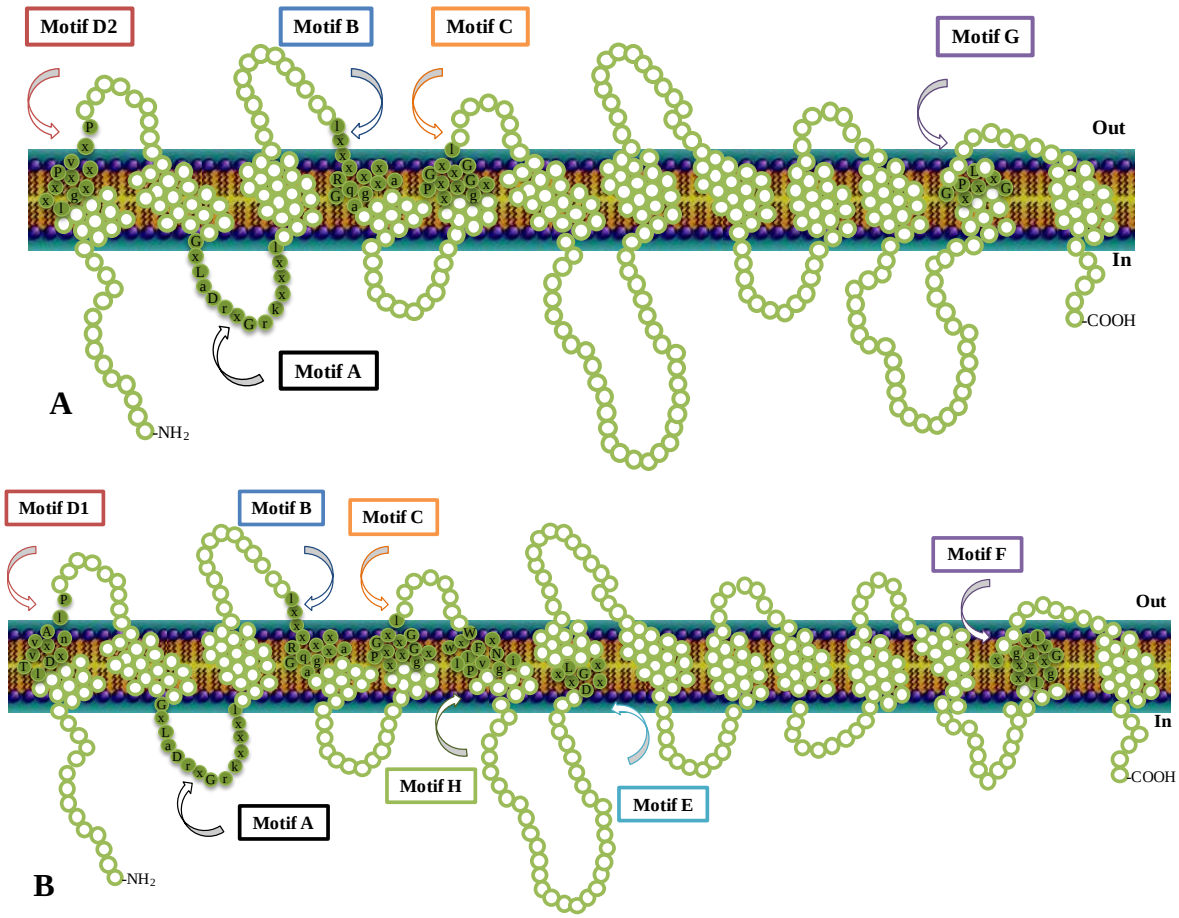


Figure 1.5. Representation of the structural model of MFS transporters with 12 TMS (A) and 14 TMS (B). The conserved motifs and respective consensus sequence are represented by full circles. An upper-letter case indicates an amino acid that occurs at a high frequency (> 70%) at a defined position, whereas a low-letter case indicates an amino acid that occurs at a low frequency (40-70%) at a defined position. The letter x stands for any amino acid [160, 173].

1.3.2. Small Multidrug Resistance (SMR) family

Transporters that belong to this family are small proteins (100-140 amino acids) that possess four TMS (Figure 1.6). They are responsible for the extrusion of lipophilic compounds, such as QACs, DNA intercalating dyes and some toxic metabolites, by an antiport mechanism [7, 8]. It has been suggested that these transporters may function as oligomeric complexes, inasmuch as some studies indicate that the *E. coli* pump EmrE functions as a tetramer [7, 127]. The efflux pumps Smr of *S. aureus*, Mmr of *M. tuberculosis*, EmrE of *E. coli* and EbrA of *Bacillus subtilis* are representatives of this family [7, 8].

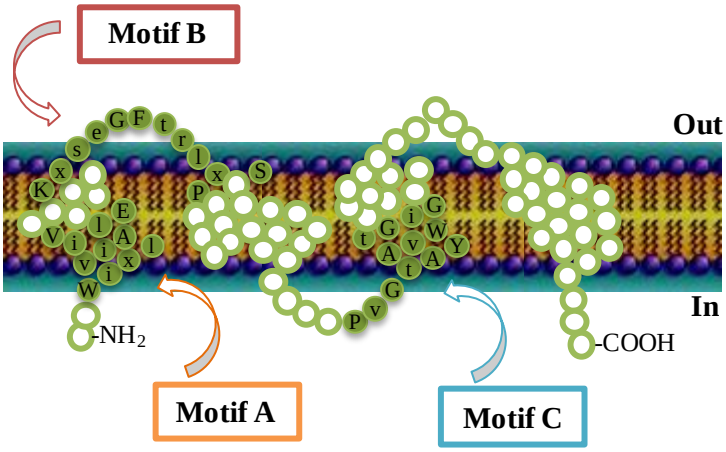


Figure 1.6. Representation of the structural model of a SMR transporter with four TMS. The conserved motifs and respective consensus sequence are represented by full circles An upper-letter case indicates an amino acid that occurs at a high frequency (> 70%) at a defined position, whereas a low-letter case indicates an amino acid that occurs at a low frequency (40-70%) at a defined position. The letter x stands for any amino acid [160, 173].

1.3.3. Multidrug and Toxic compound Extrusion (MATE) family

This family of transporters has been identified only recently hence it is not fully characterized. Sequence analysis demonstrates that bacterial MATE transporters possess a membrane topology similar to MFS transporters, with 12 TMS (Figure 1.7) [110]. They perform the extrusion of several antimicrobials by an antiport mechanism using either the proton or the sodium membrane gradient [110]. Members of this family are

the efflux pumps MepA of *S. aureus*, NorM of *Vibrio parahaemolyticus*, YdhE of *E. coli*, PmpM of *P. aeruginosa* and NorM of *Neisseria meningitidis* [110, 168].

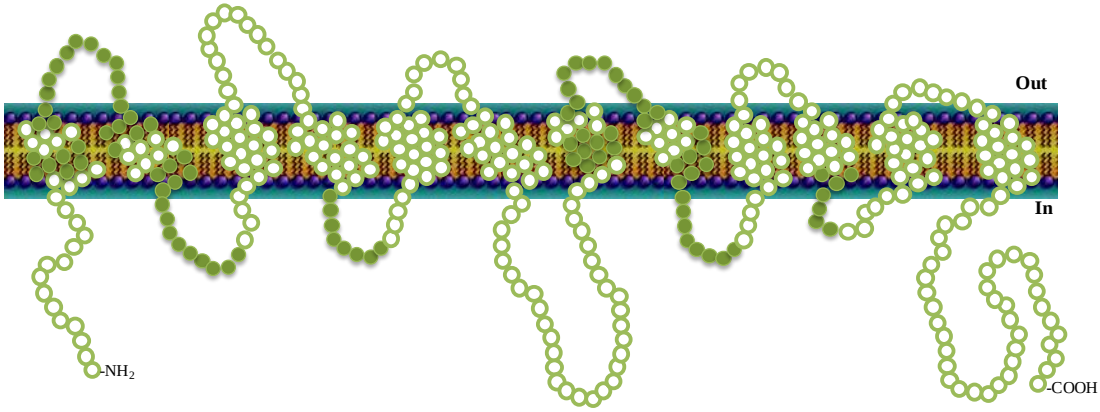


Figure 1.7. Representation of the structural model of a MATE transporter with 12 TMS. There are no conserved motifs identified to date for MATE transporters, but highly conserved sequences are represented by full circles [156].

1.3.4. Resistance-Nodulation-Cell division (RND) superfamily

RND transporters are usually found in Gram-negative bacteria, where they function as a tripartite complex [154]. The RND transporter is located in the inner membrane, presenting 12 TMS, and its periplasm domain associates with an outer membrane protein with aid of an adapter, a membrane fusion protein [154]. Some members of this superfamily are the AcrAB-TolC of *E. coli*, MexAB-OprM of *P. aeruginosa* and MtrCDE of *Neisseria gonorrhoeae* [168].

1.3.5. ATP-Binding Cassette (ABC) superfamily

The ABC superfamily comprises a large group of transporters found in both prokaryotic and eukaryotic cells that explore the free energy of the ATP hydrolysis to energize the transport of multiple substrates [103]. Examples of the bacterial representatives of the ABC superfamily are the efflux pumps Msr(A) and AbcA of *S. aureus*, LmrA of *Lactococcus lactis*, VcaM of *Vibrio cholerae* and EfrAB of *E. faecalis* [168].

1.4. Multidrug efflux pumps in *S. aureus*

Efflux-mediated resistance in *S. aureus* has been mainly associated with resistance towards fluoroquinolones and cationic compounds usually used as biocides [166, 170]. MDR efflux pumps have been identified in the chromosome and on plasmids of *S. aureus*. They all have the capacity to extrude a diverse array of antimicrobials, but while the majority of the chromosomally-encoded MDR efflux pumps extrude both fluoroquinolones and cationic compounds, plasmid-encoded MDR efflux pumps expel only cationic compounds.

Multidrug resistance is usually defined as resistance to at least one agent of three or more classes of antibiotics [128]. Though, due to the wide array of antimicrobial agents other than antibiotics that bacterial MDR efflux pumps can extrude, in this Thesis, we will use a broader definition for multidrug resistance, considering it as resistance to three or more classes of antimicrobial agents.

1.4.1. Chromosomally-encoded multidrug efflux pumps

To date, nine chromosomal MDR efflux pumps have been identified for *S. aureus* (Table 1.2). In general, their substrate specificity is somewhat superimposed, all being able to extrude fluoroquinolones and several cationic compounds, such as QACs and dyes. Nevertheless, some efflux systems can pump out other classes of antibiotics.

Table 1.2. Chromosomally-encoded MDR efflux pumps described so far in *S. aureus*.

Efflux pump	Family[a]	Regulator(s)[b]	Substrate specificity [c]	References
NorA	MFS	MgrA, NorG(?)	Fluoroquinolones (hydrophilic) QACs (e.g. TPP, benzalkonium chloride); Dyes (EtBr, rhodamine)	[88, 152, 233]
NorB	MFS	MgrA, NorG	Fluoroquinolones (hydrophilic and hydrophobic); Tetracycline QACs (e.g. TPP, cetrimide); Dyes (e.g. EtBr)	[207]
NorC	MFS	MgrA(?), NorG	Fluoroquinolones (hydrophilic and hydrophobic) Dyes (rhodamine)	[208, 213]
MepA	MATE	MepR	Fluoroquinolones (hydrophilic and hydrophobic); Glycylcyclines (tigecycline) QACs (e.g. TPP, cetrimide, benzalkonium chloride); Dyes (EtBr)	[96, 133]
MdeA	MFS	n.i.	Fluoroquinolones (hydrophilic); virginiamycin, novobiocin, mupirocin, fusidic acid QACs (e.g. TPP, benzalkonium chloride, dequalinium); Dyes (EtBr)	[82, 231]
SepA	n.d.	n.i.	QACs (e.g. benzalkonium chloride); Biguanidines (chlorhexidine); Dyes (acriflavine)	[149]
SdrM	MFS	n.i.	Fluoroquinolones (hydrophilic) Dyes (EtBr, acriflavine)	[230]
LmrS	MFS	n.i.	Linezolide; Phenicols (chloramphenicol, florfenicol); trimethoprim, erythromycin, kanamycin, fusidic acid QACs (e.g. TPP); Detergents (sodium docecyl sulphate); Dyes (EtBr)	[50]
NorD	MFS	n.i.	Not identified to date, but presumed to be similar to other Nor efflux pumps	[42]

[a] n.d.: The family of transporters to which SepA belongs has not been elucidated to date. [b] n.i.: No regulator has been identified to date for the transporter. [c] Hydrophilic fluoroquinolones: e.g., ciprofloxacin and norfloxacin; hydrophobic fluoroquinolones: e.g., moxifloxacin and sparfloxacin; QACs: quaternary ammonium compounds; EtBr: ethidium bromide; TPP: tetraphenylphosphonium bromide.

1.4.1.1. NorA

The multidrug efflux pump NorA is one of the most studied efflux systems in *S. aureus*. The chromosomal gene that codes for it, *norA*, was first described in a fluoroquinolone-resistant isolate collected in 1986 at a Japanese hospital [215]. The *norA* gene presents some genetic diversity, with three *norA* alleles described to date that differ up to 10% in the nucleotide sequence [155, 188, 197]. NorA is a 388 amino acid protein that comprises 12 TMS, belongs to the MFS and shares 44% identity with the multidrug efflux pump Bmr from *B. subtilis* [151, 233]. Several studies have shown that NorA can extrude an array of chemically and structurally dissimilar compounds, namely hydrophilic fluoroquinolones, such as norfloxacin and ciprofloxacin; dyes, like ethidium bromide and biocides, such as QACs [88, 152, 233]. It is known that *norA* basal level of expression accounts for some level of reduced susceptibility towards those antimicrobial agents [88, 89]. Increased resistance has been associated with NorA-mediated efflux via the increased expression of the *norA* gene [31, 88, 89, 153]. This increased expression can be either constitutive, through the acquisition of mutations in the *norA* promoter region, or inducible, through the action of regulatory proteins [89, 90, 94, 95]. The first mutations to be described on *norA* promoter region were point mutations occurring 89 bp upstream of the initiation codon and downstream of the -10 motif, in the 5'-UTR region [88, 153]. It has been suggested that these mutations may lead to an increase of the *norA* mRNA half-life, through an alteration of the mRNA secondary structure, which could be less sensitive to the action of RNases [55]. Other studies with strains carrying these point mutations have detected, instead, an increase in *norA* transcripts resulting from an increased rate of *norA* transcription [95]. More recently, several groups have reported the occurrence of deletions and insertions in this same region, which may lead to increased *norA* mRNA stability [31, 37, 83]. To date, no correlation was found between antimicrobial resistance and mutations occurring in *norA* coding region [155, 188]. The production of NorA may also be regulated by several regulatory systems, albeit a clear regulatory pathway remains to be elucidated (see 1.4.3.1).

Similarly to other efflux pumps belonging to the MFS, NorA uses the PMF to energize the transport of the antimicrobials across the cell membrane, via an H^+:drug antiport mechanism. Studies have shown that the NorA-mediated efflux of ethidium bromide [152] and norfloxacin [88, 153] is sensible to protonophores, such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which dissipates the membrane proton gradient. It was also demonstrated that the accumulation of norfloxacin in *E. coli* everted vesicles carrying a functional NorA was affected by the presence of nigericin, but not by valinomycin, which collapses the electric gradient, supporting the hypothesis that NorA-mediated efflux is coupled to the proton gradient across the membrane [153].

1.4.1.2. NorB

Since the first studies on NorA, evidence was gathered for the occurrence of other efflux systems in the chromosome of *S. aureus* [91, 92]. The efflux pump NorB is structurally similar to the efflux pumps Blt (41%) and Bmr (30%) from *B. subtilis*, as well as to *S. aureus* NorA (30%) and QacA (39%). It is a MFS proton-driven efflux pump encoded by the *norB* gene and composed by 463 amino acids, with 12 TMS. NorB confers resistance to some of the NorA substrates, such as hydrophilic fluoroquinolones, biocides (QACs) and to the dye ethidium bromide, as well as to non-NorA substrates, such as the hydrophobic fluoroquinolones moxifloxacin and sparfloxacin, and in a lesser extent to tetracycline [207]. A study with a mouse subcutaneous abscess model showed that NorB was important for *S. aureus* fitness, suggesting a putative role for NorB in staphylococcal pathogenesis [41]. Following studies from the same group proposed that NorB may be involved in *S. aureus* response to acid shock and reduced aeration, conditions which triggered overexpression of *norB* gene [212, 214]. In both cases, overexpression of *norB* was associated with an increased resistance to NorB substrates [212, 214]. Regulation of the efflux pump NorB is closely related to the one of NorA, involving diverse regulatory pathways (see 1.4.3.1).

1.4.1.3. NorC

The efflux pump NorC, codified by the chromosomal gene *norC*, is a 462 amino acid protein with 12 TMS that belongs to the MFS and shares 61% identity with NorB [208]. NorC is associated with low-level resistance towards hydrophilic and hydrophobic fluoroquinolones, such as ciprofloxacin, moxifloxacin and garenoxacin, and to the dye rhodamine [208, 213]. Studies have indicated that the wild-type expression of *norC* is apparently not sufficient to affect the susceptibility towards these antimicrobial agents, and that low-level resistance is achieved through *norC* overexpression [208]. NorC is under the regulation of the same regulatory pathways as NorA and NorB (see 1.4.3.1).

1.4.1.4. MepA

The efflux pump MepA was identified in studies with *S. aureus norA*$^{-}$ mutants [96]. MepA is encoded by the chromosomal gene *mepA* and it was the first multidrug transporter from the MATE family to be described in *S. aureus*. This 451 amino acid protein has 12 TMS and presents 26% and 21% identity to the MATE transporters CdeA from *Clostridium difficile* and NorM from *V. parahaemolyticus*, respectively. MepA was found to be associated with a MDR phenotype, conferring low-level resistance to QACs, such as benzalkonium chloride, cetrimide, dequalinium, tetraphenylphosphonium, to the diamidine pentamidine, to the biguanidine chlorhexidine and the dye ethidium bromide, as well as to tigecycline, an antibiotic from the class of the glycylcyclines [133]. The fluoroquinolones ciprofloxacin and norfloxacin were shown to be weak substrates of MepA [96, 133]. Recent work on mutagenesis of the MepA protein has identified some acidic residues potentially critical for the protein function, namely substrate translocation [186].

The *mepA* gene is integrated in the *mepRAB* operon. Sequence analysis revealed that MepR is similar to regulatory proteins from the MarR family but no significant similarity was found between MepB and any other protein with known function, also no association was found between MepB and MDR phenotypes [96].

1.4.1.5. MdeA

The *S. aureus* chromosomal gene *mdeA*, which encodes the efflux pump MdeA, was identified in an open reading frame expression library of the *S. aureus* genome [82]. MdeA has 479 amino acids, possesses 14 TMS and belongs to the MFS, using the PMF to energize the transport of its substrates. MdeA shares 37% identity with the efflux pump LmrB from *B. subtilis*, 24% with EmrB from *E. coli* and 23% with QacA from *S. aureus* [82]. The overexpression of *mdeA* was associated to increased resistance to the biocides benzalkonium chloride, dequalinium and tetraphenylphosphonium, to the dye ethidium bromide, and to the antibiotics virginiamycin, novobiocin, mupirocin and fusidic acid [82]. A subsequent study showed that the fluoroquinolones norfloxacin and ciprofloxacin are a weak substrate of this pump [231]. The overexpression of *mdeA* can be achieved by the occurrence of mutations in the *mdeA* promoter region, producing however only a slight increase in the MDR resistance phenotype [82].

1.4.1.6. Other chromosomally-encoded multidrug efflux pumps

The protein SepA, codified by the chromosomal gene *sepA*, has been identified as an efflux pump that confers low-level resistance to antiseptic compounds, namely benzalkonium chloride, chlorhexidine gluconate and the dye acriflavine [149]. This transporter comprises 157 amino acids and four putative TMS, a characteristic of the transporters from the SMR family. However, the conserved motifs of this family are absent from SepA, although some residues important for the transport specificity and for the antiport H^+:drug are present but in different positions, suggesting that SepA may belong to a not yet identified family of transporters [149].

SdrM is a 447 amino acid efflux pump codified by the chromosomal gene *sdrM*. SdrM shares 23% and 21% identity with the *S. aureus* MDR efflux pumps NorB and QacA, respectively. Sequence analysis suggests that SdrM, with 14 TMS, may belong to the MFS. This efflux pump was associated with low-level resistance to acriflavine, ethidium bromide and the fluoroquinolone norfloxacin [230].

More recently, Floyd and colleagues described another *S. aureus* multidrug efflux pump, LmrS (lincomycin resistance protein of *Staphylococcus aureus*), showing 39% identity with the lincomycin resistance protein LmrB of *B. subtilis*, and 25% identity with the efflux pumps FarB of *N. gonorrhoeae* and EmrB of *E. coli* [50]. LmrS was described as a 480 amino acid MFS protein, with 14 predicted TMS, being involved in increased resistance to lincomycin, linezolid, trimethoprim, chloramphenicol, tetraphenylphosphonium chloride and sodium dodecyl sulfate [50].

NorD, a putative MFS efflux pump was recently identified [42]. It is encoded by the gene *norD*, and comprises 397 amino acids. Analysis of its sequence predicts a 12 TMS protein belonging to the MFS, presenting 26% identity to NorA and NorB. Although the authors propose that NorD may share the same substrate specificity as other *S. aureus* MDR efflux pumps, no assays have been conducted to clarify this hypothesis. Nevertheless, NorD showed, in parallel with NorB, to contribute to fitness in a murine subcutaneous abscess model andto be negatively regulated by Fur, a ferric uptake regulator, in iron-restricted conditions [42].

1.4.2. Plasmid-encoded multidrug efflux pumps

The majority of *S. aureus* strains carry one or more plasmids harboring mainly determinants of resistance to antimicrobials and/or virulence [193]. Among the resistance determinants present in *S. aureus* plasmids, six MDR efflux systems have been described to date (Table 1.3) that extrude cationic compounds and dyes.

Table 1.3. Plasmid-encoded MDR efflux pumps described so far in *S. aureus*.

Efflux pump	Family	Regulator(s)[a]	Substrate specificity[b]	References
QacA	MFS	QacR	QACs (e.g. TPP, benzalkonium chloride, dequalinium) Biguanidines (e.g. chlorhexidine) Diamidines (e.g. pentamidine) Dyes (e.g. EtBr, rhodamine, acriflavine)	[17, 176]
QacB	MFS	QacR	QACs (e.g. TPP, benzalkonium chloride) Dyes (e.g. EtBr, rhodamine, acriflavine)	[162]
Smr	SMR	n.i.	QACs (e.g. benzalkonium chloride, cetrimide) Dyes (e.g. EtBr)	[67, 118]
QacG	SMR	n.i.	QACs (e.g. benzalkonium chloride, cetyltrymethylammonium) Dyes (e.g. EtBr)	[76]
QacH	SMR	n.i.	QACs (e.g. benzalkonium chloride, cetyltrymethylammonium) Dyes (e.g. EtBr)	[75]
QacJ	SMR	n.i.	QACs (e.g. benzalkonium chloride, cetyltrymethylammonium) Dyes (e.g. EtBr)	[13]

[a] n.i.: No regulator has been identified to date for the transporter. [b] QACs: quaternary ammonium compounds; TPP: tetraphenylphosphonium bromide; EtBr: ethidium bromide.

1.4.2.1. QacA/B

In the early 1980s, a gene encoding resistance to several antiseptics and disinfectants was identified on plasmid pSK1, carried by clinical isolates of *S. aureus* [203]. This gene, later designated *qacA*, encodes the efflux pump QacA that comprises 514 amino acids and is a member of the MFS presenting 14 TMS [176]. The *qacA* gene is found in large conjugative plasmids and non-conjugative multiresistance plasmids in both *S. aureus* and CoNS [114]. QacA mediates resistance to more than 30 lipophilic, mono- and divalent cations, that belong to 12 distinct chemical classes, including dyes, such as ethidium bromide and rhodamine; QACs, like benzalkonium chloride, tetraphenylphosphonium and dequalinium; diamidines, such as pentamine;

biguanidines, like chlorhexidine; and guanylhydrazones [17, 143, 204]. The transport of these substrates is driven by the PMF via an antiport H^+:drug mechanism [144, 204].

Another antiseptic resistance determinant, the gene *qacB*, has been found in plasmids isolated from strains of clinical origin dating from the 1950s [164]. The *qacB* gene encodes the efflux pump QacB and was first identified on plasmid pSK23 [126]. It is closely related to *qacA*, differing in only seven nucleotides in the entire sequence. Even so, the substrate specificity of both pumps varies, with QacB conferring resistance to only monovalent lipophilic cations [126, 162]. Mutagenesis analysis revealed that the presence of an aspartic acid at the position 323 of QacA, located within the TMS10, instead of an alanine in QacB, is critical for conveying resistance to divalent cations. Moreover, it indicates that an acidic charge in that position is essential for the binding of divalent substrates and that QacA has two different binding sites for substrates [162, 229], which is also supported by kinetic analysis of the QacA transport of monovalent and divalent fluorescent substrates [144].

Analysis of *qacA/B* genes carried by *S. aureus* clinical isolates has revealed the occurrence of some genetic variability and that the variants of either QacA or QacB may confer different levels of resistance to biocides and dyes [3]. In addition, a recent study indicated that a clinical isolate harboring a variant of QacB, which carried a glutamic acid instead of alanine in position 320, which is located in the TMS10, conferred low-level resistance to the hydrophilic fluoroquinolones norfloxacin and ciprofloxacin [148].

1.4.2.2. Smr

The efflux pump gene *smr* was identified in the late 1980s by different groups in several plasmids conferring antiseptic and ethidium bromide resistance. This gene was designated *ebr* [184], *qacC/D* [118] or *smr* [67], but sequence analysis revealed that they were identical. This gene can be found in either small non-conjugative plasmids, such as pSK89, or large conjugative and non-conjugative multiresistance plasmids, like pSK41, in both *S. aureus* and CoNS [114, 115, 118]. Sequence analysis of the *smr* gene found in several plasmids showed that it is highly conserved, with only one variant,

smr', carrying a single nucleotide alteration that results in the change of an alanine by a serine at residue 9, producing the Smr' protein. It was demonstrated that Smr' conveyed a resistance phenotype similar to Smr [74]. However, the regions flanking *smr* show some diversity, leading to the classification of *smr* cassette-like structures (comprising the *smr* gene flanked by direct repeats) in three distinct groups (types 1 to 3), apparently with no differences at the type or level of resistance conferred [2, 12, 115].

Hydropathy analysis of the product of *smr*, Smr, revealed that it has 107 amino acids, four TMS and belongs to the SMR family that uses the PMF to energize the transport of noxious compounds [67]. This efflux pump conveys low-level resistance to a narrower number of compounds when compared with QacA/B, namely monovalent cationic dyes, such as ethidium bromide, and QACs, like benzalkonium chloride [67]. Due to its small size, it was questioned if Smr, as a monomer, would be able to conduct the transport of its substrates. *In vitro* transport assays with purified Smr and site-directed mutagenesis, showed that it is capable of performing efflux, without however clarifying if as a monomer or as an oligomer [67, 161], as described for other pumps of the SMR family [7].

1.4.2.3. Other plasmid-encoded multidrug efflux pumps

Other *S. aureus* plasmid-borne efflux pumps conferring resistance to antiseptics and disinfectants include QacG, QacH and QacJ. The efflux pump gene *qacG* was identified in *S. aureus* isolates collected in the food industry. This gene was located on the 2.3 kb plasmid pST94. It encodes the efflux pump QacG that has 107 amino acids and four TMS, shares 69.2% identity with Smr and belongs to the SMR family of transporters [76]. The determinant *qacH* was first detected in a 2.4 kb plasmid isolated from a *Staphylococcus saprophyticus* strain also from the food industry. The *qacH* gene shares 76% and 70% identity to the *smr* and *qacG* genes, respectively, and encodes the efflux pump QacH, with 107 amino acid residues and four TMS that belongs to the SMR family [75]. The efflux pump gene *qacJ* was identified on a 2.65 kb plasmid found in several strains from three staphylococcal species; *S. aureus*, *Staphylococcus simulans* and *S. intermedius*, all collected from horses [13]. The encoded efflux pump QacJ also belongs to the SMR family, with 107 amino acids and four TMS, and shares

73%, 83% and 73% identity with the efflux pumps Smr, QacG and QacH, respectively. A recent report described, for the first time, the occurrence of these genes in human clinical isolates [232]. Despite the differences found between the amino acid sequences of Smr and QacG/H/J, all these pumps share almost identical substrate specificities, conferring similar levels of resistance to benzalkonium chloride, ethidium bromide and cetyltrymethylammonium bromide [13].

1.4.3. Regulation of *S. aureus* multidrug efflux pumps

1.4.3.1. Global regulators

The regulation of *S. aureus* MDR efflux pump genes is complex and affected by several global regulators that act in an intricate regulatory network and are involved in chemical and physical stress response as well as in *S. aureus* pathogenesis.

The regulator MgrA, previously named NorR or Rat, was first identified by its binding to the *norA* promoter region [53]. MgrA is highly homologous to MarR family proteins and in a lesser-extent to SarA family proteins, both transcriptional regulatory proteins that possess a helix-turn-helix motif involved in the specific binding to DNA [85, 124]. MgrA is a pleiotropic regulator that uses an oxidation-sensing mechanism [25] in the regulation of autolysis, virulence genes, antibiotic resistance genes (efflux pumps) and other genes involved in the *S. aureus* metabolism [86, 125]. This regulator was also found to modulate the expression of other global regulators, such as SarA, which affects the expression of virulence determinants [27] and the alternative sigma factor SigB, essential for the *S. aureus* chemical and stress response [11, 57]. These findings suggest that some modulation effects attributed to MgrA may be achieved in an indirect manner [125].

MgrA regulates three *S. aureus* MDR efflux pumps; NorA, NorB and NorC, as well as the non-MDR tetracycline efflux pump Tet38 and the ABC efflux pump AbcA [206, 207, 209]. Reports from different groups had described divergent effects of MgrA on the *norA* and *norB* expression in genetic backgrounds that differed in the *rsbU* locus encoding the protein RsbU from the serine/threonine phosphatase family [53, 95, 125,

206, 207]. Following studies evidenced that MgrA can be post-translationally phosphorylated by the putative serine/threonine kinase PknB [210], and that the phosphorylated MgrA-P can be dephosphorylated by RsbU [211], altering the ability of MgrA to bind to the *norA/B* promoters. A model for MgrA *norA/B* modulation was proposed after the demonstration that only MgrA was able to bind to the *norA* promoter and only MgrA-P could bind to *norB* promoter. According to this model, MgrA acts as a *norA* repressor, and upon phosphorylation, MgrA-P is released from the *norA* promoter, allowing *norA* transcription. The MgrA-P will, in turn, bind to the *norB* promoter, acting as a repressor of *norB* [211]. The cellular ratio between MgrA and MgrA-P (and hence their effect on efflux pump expression) is dependent of PknB and RsbU, which in turn are modulated by the alternative sigma factor SigB, involved in *S. aureus* stress response. Nonetheless, other yet unidentified kinases or phosphatases may also affect this cellular ratio.

It was proposed that NorC may be negatively regulated by MgrA, acting in a concerted manner with NorB. However, no MgrA recognition sites were found in the *norC* promoter region, which can suggest that MgrA may have an indirect effect over *norC* expression and that other factors may play a role in the production of this efflux pump [208].

The transcriptional regulator NorG, from the GntR-like family, was identified by its binding to the *norA* promoter in a *mgrA*$^{-}$ background [209]. It was shown that NorG was able to bind specifically to the promoters of the efflux pump genes *norA*, *norB*, *norC*, *abcA* as well as to its own promoter. No binding of NorG to the *tet(38)* or *mgrA* promoters was detected [209]. However, MgrA may bind to the *norG* promoter, thus affecting its expression [209]. Despite the binding of NorG to the promoter regions of *norA, norB, norC* and *abcA*, overexpression of *norG* was accompanied only by a mild increase in *norB* expression, suggesting that NorG is an activator of *norB* [209]. An analysis of the transcriptional profile of NorG confirmed its role as an activator of NorB and revealed that NorG affects negatively the expression of both *norC* and *abcA* [213]. It also showed that NorG activates the transcription of the global regulators *mgrA*, *arlS* and *sarZ*. SarZ, a MgrA homologue involved in the oxidative stress response of *S. aureus* was previously shown to downregulate *norB* and *tet38* expression [26]. It has been documented that NorG is absent from some prototype *S. aureus* strains, such as

MW2, MSSA476 and MRSA 252, suggesting that NorG may not be essential for efflux pump regulation in these strains [104].

The expression of NorA and NorB can be also modulated by the two-component regulatory system ArlRS, involved in adhesion, autolysis and extracellular proteolytic activity of *S. aureus* [53, 54, 116]. In addition, a recent study revealed that the expression of *norA* and the newly identified *norD* can be modulated by *fur*, a ferric uptake regulator that has a putative binding site in their promoter region [38, 42]. It also showed that NorA is iron responsive and may contribute to the export of siderophores in *S. aureus* [38].

1.4.3.2. Specific regulators

In addition to the global regulators described above, the transcription of the genes coding for some *S. aureus* MDR efflux pumps can also be modulated by specific regulators. This is the case of MepA and QacA/B, regulated by MepR and QacR, respectively. Both regulators function as sensors, binding to the substrates of the MDR efflux pumps and inducing their expression, thus acting as substrate-responsive regulators.

MepR is a self-repressive protein that binds to sequences in which pseudo palindromes are present, as well as to the motif GTTAG, both located in the promoter regions of *mepR* and *mepA*. MepR does not bind to the *mepB* promoter region [97]. The binding of MepR is stronger with the *mepA* promoter than with the *mepR* promoter, and the stoichiometry of this binding is probably different [157]. The *mepA* promoter presents both high- and low-affinity MepR binding sites that overlap with the -35 and -10 consensus sequences, resulting in a tight repression of *mepA* by MepR, which may bind as two dimers. On the other hand, the weaker and smaller MepR binding site in *mepR* promoter encompasses the transcription initiation site, located immediately downstream of the -10 consensus sequence, which can result in a weaker interaction with MepR that is thought to bind as a single dimer [109]. The weaker repression of *mepR* could explain the experimental observation that only *mepR* transcripts are detected in wild-type strains [96, 97]. Analysis of mutant strains overexpressing the

mepA gene were found to have mutations in MepR, such as the introduction of premature stop codons and consequently the production of truncated forms of MepR that can render the protein non-functional, enabling the transcription of the *mepA* gene [96, 109]. Recently, the screening of a collection of clinical strains overexpressing *mepA* revealed the occurrence of other mutations like amino acid substitutions in MepR as well as mutations in the promoter of *mepR* or *mepA* [187]. A following study has shown that some of these single-amino acid substitutions were located in the linker region connecting the dimerization and the DNA binding domains, and the high-resolution structures of MepR variants revealed conformational changes that alter their DNA binding properties [10]. It was also found overexpression of *mepA* in the absence of mutations or only substitution mutations in MepA, suggesting the existence of regulation pathways independent of MepR [186].

The auto-regulatory MepR is also responsive to the presence of MepA substrates, although the substrate specificity of MepA and MepR do not entirely overlap [97, 157]. There is evidence that MepR binds to several MepA substrates, and it is postulated that the binding of a substrate induces a change in MepR conformation so that the interaction between MepR and the DNA binding sites is diminished [109, 157].

QacR is encoded by the gene *qacR* that occurs immediately upstream of the genes *qacA* and *qacB*, being transcribed divergently to them [176]. QacR possesses a helix-turn-helix DNA binding motif, common to regulatory proteins, and belongs to the TetR family of transcriptional repressors [176]. It has been shown that QacR is a direct repressor of the expression of the *qacA* gene, by binding to the *qacA* promoter [68]. QacR binds to a large inverted repeat (IR1) that is located immediately downstream of the *qacA/B* promoters and overlaps the *qacA/B* transcription initiation sites [69]. The binding mechanism of QacR to IR1 differs from that of other TetR family of regulatory proteins, with two dimers of QacR binding cooperatively to IR1, not through protein-protein interactions but via a widened conformation of the DNA promoted by the binding of the first QacR dimer that favors the binding of the second one [190]. When bound to the IR1 in the *qacA* promoter, QacR represses the transcription of this gene [68]. However, upon addition of QacA substrates, *qacA* expression increases in a substrate concentration-dependent manner by inhibition of the QacR binding to the *qacA* promoter [68]. This inhibition is achieved by the direct interaction of QacR to the

wide array of QacA substrates [68]. Thus, QacR is also a multidrug-binding protein, for which several structures of complexes QacR:drug have been determined and have revealed the presence of several, distinct, but linked binding sites within one extended and multifaceted binding pocket that has evolved to accommodate the broadest range of noxious hydrophobic molecules [70, 189].The binding of a QacA substrate to QacR results in a change in QacR conformation, which renders the protein unable to bind IR1, thus allowing *qacA* transcription [189]. Not all compounds that are QacA substrates were shown to induce *qacA* expression or to inhibit the QacR binding to IR1, suggesting that there is a basal *qacA* expression and thus QacR is a relatively weak repressor [68].

1.5. Additional efflux pumps in *S. aureus*

Several other efflux pumps with narrower substrate specificity occur in the *S. aureus* chromosome as well as in plasmids that are associated with resistance to tetracycline, macrolides, lincosamides and/or streptogramins, among others.

Efflux pumps responsible for the extrusion of tetracycline can be found encoded in both the chromosome, *tet(38)* and in plasmids of *S. aureus*, *tet(K)* and *tet(L)*. The efflux pumps Tet38, TetK and TetL are MFS transporters and perform efflux of tetracyclines, but not minocycline and glycylcyclines [175]. As mentioned previously, the chromosomal gene *tet(38)* is one of the efflux pump genes under regulation of MgrA [207].

Resistance to macrolides, lincosamides and/or streptogramins mediated by efflux has been linked to the activity of several efflux pumps, including the MefA pump, a MFS transporter that extrudes 14-membered macrolides; the MsrA pump, a transporter of the ABC superfamily that conducts the extrusion of 14-membered macrolides and streptogramins B; the Vga efflux pumps (VgaA and variants, VgaB, VgaC and VgaE), transporters from the ABC superfamily that perfom the extrusion of streptogramins A, lincosamides and pleuromutilins [98].

The FexA efflux pump is a MFS transporter with 14 TMS that has been associated with resistance to fluorinated and non-fluorinated phenicols [98]. The *fexA* gene occurs in a non-conjugative transposon that has been detected in both plasmids and

the chromosome of *S. aureus* strains [98]. The ABC efflux pump AbcA has been described in the chromosome of *S. aureus* and linked to reduced susceptibility to β-lactams [207]. The efflux pump gene *abcA*, as *tet(38)*, is regulated by the global regulator MgrA [207].

1.6. Efflux inhibitors: a pathway to circumvent the clinical impact of efflux pumps

The recognition that efflux is an important player in resistance to antibiotics, with a proven clinical impact for several bacterial species, mainly Gram-negative bacteria, has lead to the development of strategies to overcome efflux-mediated resistance. One of such strategies relies on the design of new pharmaceuticals that could bypass efflux, such as the modification of existing antibiotics [120, 169]. Another strategy to surmount efflux activity is the inhibition of efflux pumps either directly or indirectly, using molecules known as efflux inhibitors [131]. The direct inhibition of efflux can be achieved by the blocking of the pump by a competitive or non-competitive substrate. Still, this strategy has met limited success, being less effective in pumps that possess multiple substrate-binding sites [169]. Indirect inhibition of efflux can be attained by: (i) prevention of the correct assembly of the efflux system, of particular relevance for RND pumps; (ii) disruption of the energy source; (iii) affecting the regulatory pathways that modulate the expression of efflux pumps [136, 158]. Growing interest in efflux inhibitors has lead to the identification of many molecules with the potential to inhibit efflux systems in prokaryotic and eukaryotic cells. These non-antibiotic molecules could be used as "helper compounds" in therapy, that is, as compounds that when co-administered with an antibiotic would restore the activity of the latter by suppressing its efflux [106, 107, 132]. As a result, efflux inhibitors could "redeem" the therapeutic value of antibiotics to which a certain bacteria is resistant via efflux.

The recognition that some compounds known as efflux inhibitors of MDR efflux pumps have affinity for pump substrates, originating an inhibitor-substrate complex [235], revealed an alternative route to overcome efflux, the design of improved "escort

molecules" that may complex to the substrates and deliver them into the cell, preventing their efflux [174].

Many efflux inhibitors of *S. aureus* MDR efflux systems have been identified by screening of molecules known as inhibitors of other, mainly eukaryotic, efflux systems (e.g, reserpine and verapamil); screening of non-antibiotic compounds already used in the clinical practice for the treatment of conditions other than bacterial infections (e.g., the antipsychotic agents phenothiazines) and by the screening of large chemical libraries [131, 185].

One of the first molecules described with activity as efflux inhibitor in *S. aureus* was reserpine, an indole alkaloid that occurs in the plant *Rauwolfia serpentine* and that has been used clinically as an antihypertensive and neuropleptic [150]. It was first identified as an efflux inhibitor of the eukaryotic P-gp pump and afterwards of the Bmr pump of *B. subtilis,* an homolog of the pump NorA [151]. Later on, it was showed to inhibit NorA efflux in prototype strains as well as in clinical isolates [1, 152].

The inhibitor verapamil is a blocker of calcium channels used clinically as an antiarrhythmic agent. It has been described as an inhibitor of the eukaryotic P-gp pump [234]. It was later demonstrated to be an inhibitor of the NorA efflux pump [1]. Other P-gp inhibitors have also been demonstrated to have the capacity to inhibit efflux in *S. aureus*, such as GG918 [58], biricodar and timcodar [147].

Phenothiazines, like thioridazine and chlorpromazine, are a class of compounds widely used as antipsychotic agents. They have been shown to possess antimicrobial activity against bacteria, protozoa (*Plasmodium falciparum* and *Leishmania*), virus and prions [4], as well as the ability of plasmid curing [199]. Additionally, several studies report their activity as inhibitors of efflux in *S. aureus* [31, 93].

Many natural compounds have also been tested for their efflux inhibitory activity, and several were found to affect efflux activity in *S. aureus* [185], including the flavonoid 5'-MHC [201], the alkaloid piperine [99, 142], indoles [183], thiophenes [22], *N*-caffeoylphenalkylamides [141] and polyacylated oligosaccharides [165].

Besides their role as efflux inhibitors, several of these molecules are used in the research field to screen for efflux activity both in laboratory-derived strains and in strains of clinical origin [218].

1.7. Dissertation Objectives and Outline

As demonstrated throughout the Introduction, efflux systems capable of extruding a given antibiotic or a plethora of different antimicrobials may play an important role in the establishment and survival of bacteria in the clinical setting. However, among the several mechanisms of bacterial resistance to these agents, efflux is one of the least characterized to date, in particular for Gram-positive bacteria.

The main objective of this Dissertation was to study efflux-mediated resistance to different antimicrobial agents, which included antibiotics and biocides, in *S. aureus*. The specific objectives were the following: (i) to evaluate the role played by three major *S. aureus* MDR efflux pumps in resistance towards different antimicrobial agents; (ii) to assay the contribution of efflux on resistance towards antibiotics and biocides in *S. aureus* clinical strains; (iii) to ascertain the temporal response of representative *S. aureus* strains to different substrates of efflux systems and its correlation with the development of antimicrobial resistance phenotypes.

Accordingly, this Dissertation is structured as follows:

Part I: Characterization of the physiological role of *S. aureus* multidrug efflux pumps

Chapters 2 to 4 comprise a revision of the role of several *S. aureus* MDR efflux pumps on antimicrobial resistance in reference and clinical *S. aureus* strains, namely the chromosomally-encoded NorA (Chapter 2) and the plasmid-encoded pumps QacA (Chapter 3) and Smr (Chapter 4).

Part II: Efflux-mediated resistance to antimicrobial agents in *S. aureus*

Chapters 5 and 6 focus on the contribution of the main *S. aureus* MDR efflux pumps on the resistance to fluoroquinolones (Chapter 5) and biocides (Chapter 6) in a collection of ciprofloxacin-resistant *S. aureus* clinical strains.

Part III: Efflux as an important player in the emergence of antimicrobial resistance in *S. aureus*

Chapter 7 is dedicated to the examination of the involvement of efflux on the development of antimicrobial resistance, including the interplay between efflux and the acquisition of mutations, in a reference and two ciprofloxacin-resistant clinical strains.

Part IV: General Discussion and Conclusions

Chapter 8 presents a general discussion of the findings obtained in this Dissertation, scrutinizing the results obtained and contextualizing within literature. Chapter 9 includes the closing remarks and indications for future studies.

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CHAPTER 2

Revising the role of NorA

2.1. The role of NorA in the adaptative response of *Staphylococcus aureus* to stress stimuli

2.2. The genetic diversity of the *norA* gene

Summary

NorA was the first *S. aureus* chromosomally-encoded efflux pump to be described. Despite the later description of additional MDR efflux systems showing partially superimposed substrate specificity, NorA remains the prototype system used in studies on efflux-mediated resistance in *S. aureus*.

This Chapter describes the work carried out aiming at a better understanding of the role of this pump in resistance to antimicrobials in *S. aureus*. In a first approach (sub-Chapter 2.1), we evaluated the transient nature of the *norA*-mediated response to ethidium bromide (EtBr) by strain *S. aureus* ATCC25923. The work developed demonstrated the important role of NorA in the long-term response of *S. aureus* to chemical stress. It also revealed that the increased expression of the *norA* gene previously reported for the EtBr-adapted strain ATCC25923$_{EtBr}$ resulted from an increase in the transcription rate of this gene instead of an increase in *norA* mRNA stability. Additionally, evidence suggests that the increased expression rate of *norA* may be due to a combined effect of a deletion in the promoter region of this gene and the activity of the regulator MgrA. In a second approach (sub-Chapter 2.2), the genetic variability of the *norA* gene was explored by analyzing the *norA* alleles present in a collection of 52 ciprofloxacin-resistant *S. aureus* clinical isolates. Phylogenetic analysis showed that *norA* is a conserved gene, yet two alleles were found, *norAI* and *norAII*, of which *norAI* was the predominant one, in line with previous findings. Although the association between *norA* alleles and susceptibility profiles was difficult to ascertain, *norAI* may be linked to a mild increase in resistance towards fluoroquinolones and some biocides. Several mutations were found in the *norA* promoter region, but no overexpression could be detected, apart from an increased expression level of a mutated *norAI* in the presence of ciprofloxacin. The screening of the *norA* structural gene also detected mutations originating altered NorA proteins, with NorAI variants associated with higher resistance levels than NorAII in the strains in study.

2.1. The role of NorA in the adaptative response of *Staphylococcus aureus* to stress stimuli

2.1.1. Introduction

In a previous work from our group, a fully-susceptible reference strain, *S. aureus* ATCC25923, was challenged by a step-wise exposure to EtBr, a substrate common to most MDR efflux pumps, yielding an EtBr-adapted strain, ATCC25923$_{EtBr}$ [6, 7]. Characterization of ATCC25923$_{EtBr}$ revealed that it had developed a phenotype of reduced susceptibility to fluoroquinolones, several biocides and dyes. Gene expression analysis showed that this was the result of an efflux-mediated response, via overexpression of the *norA* efflux pump gene. Sequence analysis of the *norA* promoter showed a 70 bp deletion in this region that could have triggered the increased expression level of *norA*. In the present work, we aimed to further scrutinize the efflux-mediated response of ATCC25923$_{EtBr}$, by subjecting this strain to a reversion process (successive growth in EtBr-free media). This process resulted in a reverted strain, ATCC25923$_{EtBr_rev}$ presenting a susceptibility phenotype similar to the original ATCC25923 strain, indicating that the phenotype observed for strain ATCC25923$_{EtBr}$ was a transient one, fully reversible and thus, not mediated by a stable alteration (mutation).

2.1.2. Material and Methods

Strains. ATCC25923$_{EtBr}$ was originated by serial passage of the reference, pan-susceptible strain ATCC25923, in tryptone soya broth (TSB, Oxoid Ltd., Basingstoke, UK) medium supplemented with increasing concentrations of EtBr. ATCC25923, with an EtBr MIC of 6.25 mg/L, was successively grown in increasing concentrations of EtBr (5, 10, 20, 40, 60, 80 and 100 mg/L) until the adapted strain ATCC25923$_{EtBr}$ was obtained, with an EtBr MIC of 200 mg/L. The entire adaptation process took 82 days [6, 7, 10]. Strain ATCC25923$_{EtBr}$ presents a reduced susceptibility profile to fluoroquinolones, biocides and dyes, associated with overexpression of the *norA* efflux

pump gene [6, 7]. All cultures were grown in TSB or tryptone soya agar (TSA, Oxoid) at 37ºC, which were supplemented with 50 mg/L of EtBr for ATCC25923$_{EtBr}$. For MIC determinations all strains were cultured in Mueller-Hinton broth (MHB, Oxoid).

Reversion procedure. The EtBr-adapted strain ATCC25923$_{EtBr}$ was sequentially grown in TSB medium free of EtBr until a culture showing a reversion of the reduced susceptibility phenotype to EtBr was achieved. This process was carried out during 84 days and yielded a reverted culture denominated ATCC25923$_{EtBr_rev}$ [6, 10].

Chemical compounds. Antibiotics, biocides and dyes were acquired in powder form from several sources, as follows: EtBr, tetraphenylphosphonium bromide, dequalinium chloride, pentamidine isothionate salt, berberine, benzalkonium chloride, cetrimide, ciprofloxacin, norfloxacin, levofloxacin, sparfloxacin, rifampicin (Sigma, St. Louis, MO, USA), chlorhexidine diacetate (Fluka Chemie GmbH, Buchs, Switzerland). The efflux inhibitor verapamil was acquired from Sigma. All solutions were prepared in the day of the experiment in desionized water.

Drug susceptibility testing. Susceptibility to fluoroquinolones, biocides and dyes of the strains in study was established by MIC determination using the two-fold broth microdilution method, according to the CLSI guidelines [4]. Cultures were grown overnight in MHB, at 37ºC. A cellular suspension was prepared in phosphate buffered saline (PBS) with a turbidity of 0.5 in the McFarland standard, and 0.02 mL were then used to inoculate each well of a 96-well plate containing two-fold dilutions of each antimicrobial agent to be tested in MHB. The plates were then incubated at 37ºC for 18 h and the MIC values, corresponding to the lowest concentration of compound that inhibited visible growth, were recorded. MICs for antibiotics were evaluated according to the CLSI guidelines [4]. Each MIC determination was carried out in triplicate.

Evaluation of efflux activity. A semi-automated fluorometric method was used for assessment of EtBr efflux activity in the strains in study [25]. Briefly, cultures were grown in TSB medium at 37ºC with shaking until an optical density at 600 nm (OD_{600}) of 0.6, cells were collected by centrifugation at 13,000 rpm for 3 min and washed twice with 1X PBS. The cellular suspension was then adjusted to an OD_{600} of 0.3 and incubated with EtBr (0.25 mg/L for ATCC25923 and ATCC25923$_{EtBr_rev}$, 2 mg/L for ATCC25923$_{EtBr}$) plus 200 mg/L of verapamil (the most active efflux inhibitor). After 60

min of incubation, the EtBr loaded cells were collected by centrifugation at 13,000 rpm for 10 min and resuspended in PBS to an OD_{600} of 0.6. Aliquots of 0.05 mL of this cellular suspension were added to 0.2 mL microtubes containing: i) 0.05 mL of glucose 0.8% (condition for maximum of efflux) or ii) 0.05 mL of 400 mg/L verapamil (condition for minimum of efflux), for the final concentrations in the assays of 0.4% glucose, 200 mg/L verapamil and a cellular suspension at OD_{600} 0.3. Efflux assays were then conducted in a Rotor-Gene 3000™ thermocycler using real-time analysis software (Corbett Research, Sidney, Australia). The fluorescence of EtBr was measured at the end of every cycle of 10 sec for 10 min, at 37ºC. The raw data obtained was then normalized against data from non-effluxing cells at each point, which were considered to be the maximum fluorescence values that could be obtained during the assay. The relative fluorescence thus corresponds to the ratio of fluorescence that remains per unit of time relative to the EtBr loaded cells.

Macrorestriction analysis. Cultures were typed by pulsed-field gel electrophoresis (PFGE) analysis, using well-established protocols. Briefly, agarose disks containing intact chromosomal DNA were prepared as previously described [3] and restricted with SmaI, according to the manufacturer's recommendations (New England Biolabs, Ipswich, MA, USA). Restriction fragments were then resolved by PFGE, which was carried out in a contour-clamped homogeneous electric field (CHEF) apparatus (CHEF-DRII, Bio-Rad, Hercules, CA, USA) as previously described [3]. Lambda ladder PFG marker (New England Biolabs) was used as a molecular weight marker.

Gene expression analysis by RT-qPCR. Analysis of the expression level of efflux pump genes and of the global regulator gene *mgrA* was done by quantitative RT-PCR, using a QuantiTect SYBR Green RT-PCR Kit (QIAGEN, Hilden, Germany). The primers used are described in Table 2.1. Total RNA was isolated from cultures previously treated with RNAprotect bacteria (QIAGEN), using the RNeasy Mini Kit (QIAGEN) following the manufacturer instructions and treated on-column with DNase I RNase-free (QIAGEN) for two hours. The relative quantity of mRNA of each gene in the EtBr-adapted and reverted strains was determined by the comparative threshold cycle (C_T) method [18] by comparison of the respective mRNA quantity in the original ATCC25923 strain. The assays were conducted in a Rotor-Gene 3000™ with real-time analysis software (Corbett Research). The method was applied to the analysis of three

assays conducted with three independent total RNA extractions. Controls for genomic DNA contamination were used. 16S rDNA was used as a reference control.

Amplification and sequencing of the *norA* gene and its promoter region. The entire *norA* gene and its promoter region were amplified by PCR and sequenced using three pairs of primers, norA1, norA2, and norA3 (Table 2.1). The reaction mixture (0.05 mL) contained 2.5 U of Taq DNA Polymerase (Fermentas Inc., Ontario, Canada), 1X Taq buffer (Fermentas), 30 pmol of each primer, 0.2 mM of dNTPs (GE Heathcare, Buckinghamshire, UK) and 1.75 mM $MgCl_2$. All PCRs were performed in a thermocyler Mastercycler personal 5332 (Eppendorf AG, Hamburg, Germany). The conditions for amplification were as follows: an initial DNA denaturation step of 3 min at 95ºC, followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 52ºC (norA1), 45ºC (norA2), 50ºC (norA3) for 1 min and extension at 72ºC for 1 min, followed by a final extension step at 72ºC for 5 min. All PCR products were analysed by 1% agarose gel electrophoresis, purified and sequenced in both strands with the same set of primers. Sequences were analysed and aligned using the free software programs BioEdit v. 7.0.9.0 (www.mbio.ncsu.edu/BioEdit/bioedit.html) and ClustalW2 (www.ebi.ac.uk/Tools/clustalw2/index.html), respectively.

Determination of initiation transcription sites by 5'-RACE PCR. The *norA* initiation transcription site was determined for strains ATCC25923 and $ATCC25923_{EtBr}$. Total RNA was isolated as described for gene expression assays. Total RNA was then used as template for 5'-RACE PCR using the 5'/3' RACE Kit, 2nd generation (Roche Diagnostics GmbH, Mannheim, Germany), as per the manufacturer's instructions. Briefly, a first-strand cDNA was synthesized using the primer SP1 (Table 2.1). cDNA was then purified with High Pure PCR Product Purification Kit (Roche Diagnostics) using the protocol described in the 5´/3' RACE Kit. A 3' poli-A tail was added to the purified cDNA, and the poli-A cDNA was amplified by a nested PCR using SP2 as an outer primer and SP3 as an inner primer (Table 2.1). The Expand High Fidelity^{Plus} PCR System (Roche Diagnostics) was used for the nested PCR. All other primers and enzymes were provided by the 5'/3' RACE Kit. The products were analysed in a 3.5% Metaphor agarose (Lonza, Basel, Switzerland) gel electrophoresis, purified and sequenced using primer SP3. Sequences were analysed with the program BioEdit.

Determination of *norA* mRNA half-life. The half-life of the *norA* mRNA was determined for the parental strain ATCC25923 and its adapted counterpart $ATCC25923_{EtBr}$. Strains were grown overnight in 10 mL of TSB medium at 37ºC with shaking. A volume of 200 mL of TSB was inoculated with 1 mL of the overnight culture and incubated at 37ºC with vigorous shaking until an OD_{600} of 0.3-0.4 was reached. At this point, RNA synthesis was arrested by addition of 200 mg/L of rifampicin (RIF). At specific time points after RIF addition (0, 2.5, 5, 7.5, 10, 12.5, 15 and 20 min), an aliquot of 11 mL was taken, of which 10 mL were added to 10 mL of a ice-cold acetone:ethanol (1:1) mixture and kept immediately at -80ºC. The remaining 1 mL was used for monitoring cell viability, for which serial dilutions were prepared in PBS and plated in TSA and TSA supplemented with 200 mg/L RIF and incubated overnight. The number of colony forming units per mL (cfu/mL) was determined for each time point analysed. The occurrence of growth in the presence of RIF evidenced that RNA synthesis was not fully arrested and the experiment discarded. For RNA isolation using the Trizol method [2], the mixture of the cells kept in acetone:ethanol at -80ºC was thawed on ice, cells were harvested by centrifugation at 10,000 rpm for 15 min and the cells were lyzed in 0.25 mL of TE 1X plus 10 mg/mL of lysozyme and 0.2 mg/mL of lysostaphin followed by a 5 min incubation at room temperature (RT). To each cell suspension, 1 mL of TRI reagent (Sigma) was added and the misture was homogeneized. After 5 min incubation at RT, 0.2 mL of chloroform was added and the mixture was vigorously shaken for 15 sec followed by 2 min incubation at RT. The mixture was centrifuged at 13,000 rpm for 15 min at 4ºC for phase separation and the aqueous phase containing the RNA was collected. For RNA precipitation, 0.5 mL of isopropanol was added to the RNA aqueous phase and kept overnight at – 20ºC. The RNA was then collected by centrifugation at 13,000 rpm for 10 min at 4ºC and the pellet washed with 70% ethanol. The pellet was air-dried for 5-10 min and resupended in RNase-free water. The RNA solution was then incubated at 55ºC for 10 min and treated with DNase I RNase-free (QIAGEN), following the manufacturers instructions. The RNA solution was then cleaned with the RNA clean-up protocol of the RNeasy Mini Kit (QIAGEN) and quantified spectrofotometrically using a NanoDrop 1000 apparatus (Thermofisher, Waltham, MA, USA). RNA integrity was verified by electrophoresis in a 1% agarose – 2.2 M formaldehyde gel. The half-life of the *norA*

mRNA was determined by RT-qPCR, using the same amount of total RNA template, by calculation of the relative quantity of *norA* mRNA at each time point relatively to the initial point of addition of RIF (t = 0) by the comparative threshold cycle (C_T) method [18] in a Rotor-Gene 3000™. For determination of the decay of the mRNA, it was considered that the maximum quantity of *norA* mRNA is observed at t = 0. The primers used for determination of *norA* mRNA half-life are listed in Table 2.1.

Table 2.1. Primers used in this study.

Primer	Sequence (5'-3')	Amplicon Size (bp)	Reference
For RT-qPCR and norA mRNA half-life determination			
norA_Fw	TTCACCAAGCCATCAAAAAG	95	[20]
norA_RT(Rv)	CCATAAATCCACCAATCCC		[5]
norB_Fw	AGCGCGTTGTCTATCTTTCC	213	[7]
norB_Rv	GCAGGTGGTCTTGCTGATAA		
norC_Fw	AATGGGTTCTAAGCGACCAA	216	[7]
norC_Rv	ATACCTGAAGCAACGCCAAC		
mepA_RT(Fw)	TGCTGCTGCTCTGTTCTTTA	198	[7]
mepA_RT(Rv)	GCGAAGTTTCCATAATGTGC		
mdeA_RT(Fw)	GTTTATGCGATTCGAATGGTTGGT	155	[13]
mdeA_RT(Rv)	AATTAATGCAGCTGTTCCGATAGA		
mgrA_RT(Fw)	GGGATGAATCTCCTGTAAACG	131	This study
mgrA_RT(Rv)	TTGATCGACTTCGGAACG		
16S_27f	AGAGTTTGATCMTGGCTCAG	492	[17]
16S_519r	GWATTACCGCGGCKGCTG		
For conventional PCR			
norA1_Fw	TGTTAAGTCTTGGTCATCTGCA	761	This study
norA1_Rv	CCATAAATCCACCAATCCC		
norA2_Fw	TTCACCAAGCCATCAAAAAG	620	[5]
norA2_Rv	CTTGCCTTTCTCCAGCAATA		
norA3_Fw	GGTCATTATTATATTCAGTTGTTG	419	This study
norA3_Rv	GTAAGAAAAACGATGCTAAT		[19]
For 5'-RACE PCR			
SP1	GTACATCAAATAACGCACC		This study
SP2	CCATAAATCCACCAATCCC		This study
SP3	AGCAGCAACAAGTAACCCTAAA		This study

Fw: forward; Rv: reverse; M: A/C; W: A/T; K: G/T.

2.1.3. Results

2.1.3.1. Reversal of the reduced susceptibility phenotype by ATCC25923$_{EtBr_rev}$

The EtBr-adapted strain, ATCC25923$_{EtBr}$ [7], was submitted to serial passages in EtBr-free medium. This process yielded a culture with an EtBr MIC of 1.5 mg/L that was designated ATCC25923$_{EtBr_rev}$ (Table 2.2). The SmaI macrorestriction patterns of the original and the two derivative strains were analyzed by PFGE and no differences were found, indicating that no contamination occurred during the reversion process (Figure 2.1).

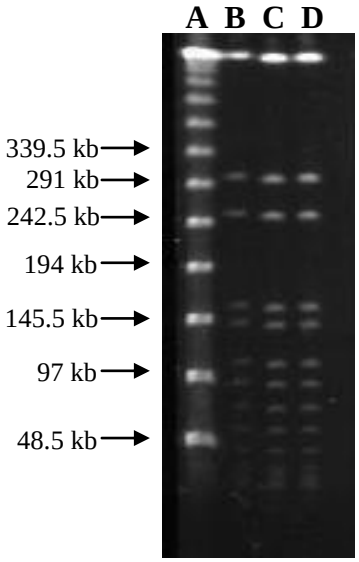

Figure 2.1. SmaI macrorestriction profiles of strains ATCC25923 and its derivatives, ATCC25923$_{EtBr}$ and ATCC25923$_{EtBr_rev}$ for contamination control throughout the processes of adaptation to EtBr and reversion. A: lambda ladder PFG marker; B: ATCC25923; C: ATCC25923$_{EtBr}$; D: ATCC25923$_{EtBr_rev}$.

The susceptibility profile of the reverted strain towards antimicrobial compounds known as NorA substrates was evaluated by MIC determination (Table 2.2). Comparison of the susceptibility profiles of the three strains revealed that ATCC25923$_{EtBr_rev}$ lost the reduced susceptibility phenotype presented by ATCC25923$_{EtBr}$ not only to EtBr but also to all the biocides tested as well as to fluoroquinolones. In fact, the MIC values presented by ATCC25923$_{EtBr_rev}$ were lower

two- to 256-fold relatively to ATCC25923$_{EtBr}$. This reduction was more pronounced for EtBr and pentamidine. The MIC values of fluoroquinolones also suffered a reduction for ATCC25923$_{EtBr_rev}$ relatively to ATCC25923$_{EtBr}$, ranging from 2-fold for sparfloxacin to 16-fold for the more hydrophilic ciprofloxacin and norfloxacin. Moreover, ATCC25923$_{EtBr_rev}$ presented, overall, lower MIC values than the ones of the parental strain ATCC25923.

Table 2.2. MIC (mg/L) values of biocides, dyes and fluoroquinolones for ATCC25923 and its derivatives ATCC25923$_{EtBr}$ and ATCC25923$_{EtBr_rev}$. Values in brackets represent the increase/decrease in the MICs for ATCC25923$_{EtBr}$ and ATCC25923$_{EtBr_rev}$ in comparison with the original ATCC25923 and the EtBr-adapted strain, respectively.

Antimicrobial compound	MIC (mg/L)		
	ATCC25923	ATCC25923$_{EtBr}$	ATCC25923$_{EtBr_rev}$
Biocides and dyes			
EtBr	6.25	200 (↑ 32x)	1.5 (↓ 128x)
BER	200	>800 (↑ 4x)	25 (↓ 32x)
BAC	0.75	3 (↑ 4x)	0.18 (↓ 16x)
CET	2	8 (↑ 4x)	0.125 (↓ 64x)
TPP	12.5	100 (↑ 8x)	6.25 (↓ 16x)
DQ	6.25	50 (↑ 8x)	0.75 (↓ 64x)
PT	50	200 (↑ 4x)	0.75 (↓ 256x)
CHX	0.375	0.75 (↑ 2x)	0.375 (↓ 2x)
Fluoroquinolones			
CIP	0.25	1 (↑ 4x)	0.064 (↓ 16x)
NOR	0.5	2 (↑ 4x)	0.125 (↓ 16x)
LEV	0.25	0.5 (↑ 2x)	0.125 (↓ 4x)
SPX	0.125	0.125 (-)	0.064 (↓ 2x)

EtBr: ethidium bromide; BER: berberine; BAC: benzalkonium chloride; CET: cetrimide; TPP: tetraphenylphosphonium bromide; DQ: dequalinium chloride; PT: pentamidine isothionate salt; CHX: chlorhexidine diacetate; CIP: ciprofloxacin; NOR: norfloxacin; LEV: levofloxacin; SPX: sparfloxacin.

2.1.3.2. Reverted phenotype of ATCC25923$_{EtBr_rev}$ is linked to a reduced efflux activity

The capacity of each strain to efflux EtBr was evaluated by real-time fluorometry. Strain ATCC25923$_{EtBr_rev}$ showed a profile of EtBr efflux similar to the one of the parental strain ATCC25923 (Figure 2.2). In particular, both ATCC25923 and ATCC25923$_{EtBr_rev}$ showed a slow efflux of EtBr, with a loss of 40% of fluorescence over the 10-min period of the assay, whereas the adapted strain ATCC25923$_{EtBr}$ had a prompt efflux activity, with a loss of 60% of fluorescence during the first minute of the assay (Figure 2.2).

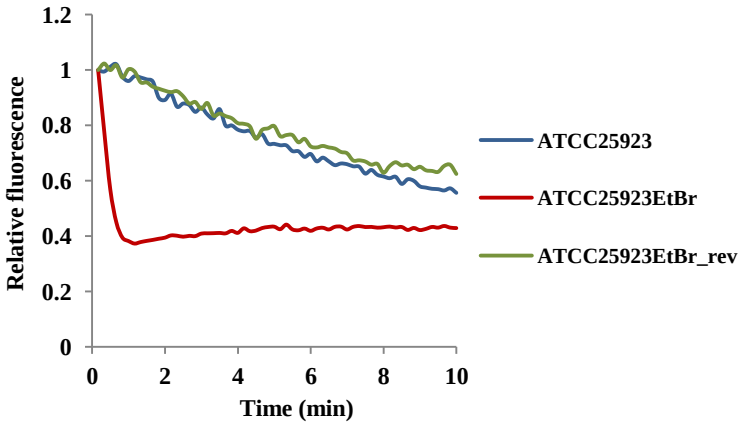

Figure 2.2. Assessment of efflux activity by real-time fluorometry for ATCC25923 (blue) and its derivatives ATCC25923$_{EtBr}$ (red) and ATCC25923$_{EtBr_rev}$ (green). The data presented was normalized against the data obtained in conditions of no efflux (absence of glucose and presence of 200 mg/L of the efflux inhibitor verapamil).

Altogether, these results suggest that the higher susceptibility to biocides, dyes and fluoroquinolones presented by ATCC25923$_{EtBr_rev}$ is associated to a reduced capacity of this strain to efflux these compounds.

2.1.3.3. Gene expression analysis

Previous work with the EtBr-adapted strain ATCC25923$_{EtBr}$ revealed that the reduced susceptibility profile was due to an increased expression of the *norA* gene and

thus a potential increased activity of NorA. The expression level of efflux pump genes coding for the main chromosomally-encoded efflux pumps *norA*, *norB*, *norC*, *mepA*, *mdeA* and *sepA* was studied for ATCC25923$_{EtBr_rev}$ and compared to the previous data obtained for ATCC25923$_{EtBr}$. In addition, the level of expression of *mgrA*, encoding the global regulator MgrA, was also assessed for both strains (Figure 2.3).

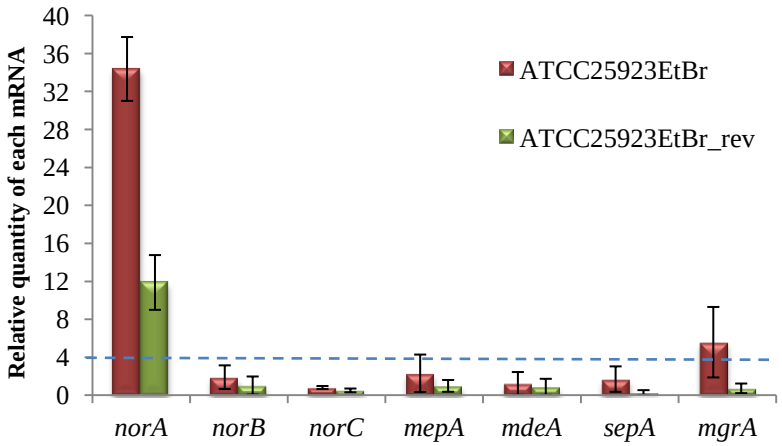

Figure 2.3. Quantification of the expression level of efflux pump genes and of the regulator gene *mgrA* for the adapted ATCC25923$_{EtBr}$ and reverted ATCC25923$_{EtBr_rev}$ strains. The expression level of each gene was calculated using the comparative threshold (C_T) method [18], by comparing the expression level of each gene in strains ATCC25923$_{EtBr}$ and ATCC25923$_{EtBr_rev}$ relatively to the parental strain ATCC25923, after normalization with the reference 16S rDNA. The assays were performed in triplicate. Overexpression was considered to occur for values ≥ 4 [5, 7].

As described previously [7], the *norA* gene is overexpressed in ATCC25923$_{EtBr}$ (34.38 ± 3.36) relatively to the parental strain. In this study, we further observed that the *norA* overexpression in ATCC25923$_{EtBr}$ was accompanied by overexpression of the regulator gene *mgrA* (5.59 ± 3.71). Analysis of the data for the reverted strain ATCC25923$_{EtBr_rev}$ showed a decrease in the expression level of both *norA* and *mgrA*, although *norA* remained overexpressed (11.89 ± 2.88) (Figure 2.3). A slight decrease was also observed for the remaining genes tested. A reduction in the expression level of the two genes was expected for ATCC25923$_{EtBr_rev}$ on the basis of its observed phenotype. However, *norA* remained overexpressed in this strain, suggesting that other factors may affect NorA production or activity.

2.1.3.4. Analysis of the *norA* promoter region and *norA* mRNA stability

The expression of *norA* is multifactorial, being attributed not only to the regulation by MgrA [11, 22, 23], but also to the occurrence of mutations and deletions/insertions in the promoter region [8, 12]. Previous findings have shown that a 70 bp deletion had occurred in the promoter region of the *norA* gene of ATCC25923$_{EtBr}$, more precisely immediately downstream of the -10 consensus sequence and encompassing the majority of the 5'-UTR [7]. In addition, the transcription initiation site assigned for the *norA* gene, an adenine located at the position -93 and distanced from the -10 motif by 6 nucleotides [12], was also comprised in the 70 bp fragment that was deleted (Figure 2.4). The new transcription initiation site of *norA* for ATCC25923$_{EtBr}$ was determined by 5'-RACE PCR. This approach allowed us to assign the initiation of transcription to a timine at position -22, and to verify that the distance of this site to the -10 motif was of 7 nucleotides, similar to the one of ATCC25923 *norA* (Figure 2.4).

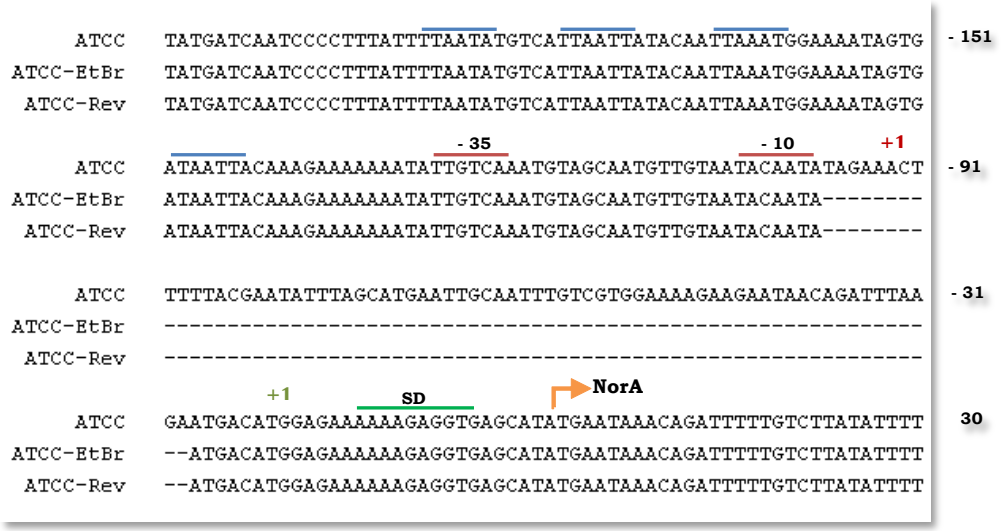

Figure 2.4. Alignment of the *norA* promoter region of ATCC25923 (ATCC), ATCC25923$_{EtBr}$ (ATCC-EtBr) and ATCC25923$_{EtBr_rev}$ (ATCC-Rev) strains. Blue lines: sequences recognized by the regulator MgrA; red lines: consensus promoter sequences -35 and -10; green line: Shine-Dalgarno sequence; +1: transcription initiation sites (in red: ATCC25923; in green: ATCC25923$_{EtBr}$ and ATCC25923$_{EtBr_rev}$); orange arrow: start of *norA* coding region.

It has also been proposed that alterations in the *norA* promoter region may increase the stability of the *norA* mRNA [8, 12]. To test this hypothesis, the *norA*

mRNA half-life was determined for both the parental and EtBr-adapted strain. A small difference was encountered, with the *norA* mRNA half-life of ATCC25923 being of 3.5 min and that of ATCC25923$_{EtBr}$ of 2.9 min. Thus, in opposition to what was expected, the stability of the *norA* mRNA suffered a mild reduction in ATCC25923$_{EtBr}$.

In sum, these findings indicate that the mechanism underlying the increased level of *norA* mRNA in ATCC25923$_{EtBr}$ is associated to an increased transcription rate of the gene instead of an increased stability of the *norA* mRNA.

2.1.3.5. Genetic analysis of the *norA* structural gene

To date, a few alterations in the *norA* coding region have been described in literature, but their impact on the NorA activity is still unknown. To verify the occurrence of such mutations, the *norA* coding region was sequenced for the three strains in study. The alignment of the NorA sequences of the parental and derivative strains revealed that NorA suffered the mutation Phe-303→ Tyr in the adapted strain ATCC25923$_{EtBr}$. Surprisingly, the NorA protein of ATCC25923$_{EtBr_rev}$ presented a reversion of this mutation, with NorA showing a phenylalanine at position 303. Most importantly, ATCC25923$_{EtBr_rev}$ *nor*A presented a stop codon at position 274, thus producing a truncated NorA protein that may have an impaired activity.

2.1.4. Discussion

2.1.4.1. The role of NorA on short-term vs. long-term response to stimuli

Previous work from our group demonstrated that the continuous exposure of an antibiotic-susceptible reference strain, *S. aureus* ATCC25923, to increasing concentrations of EtBr, a substrate common to the majority of the *S. aureus* MDR efflux pumps, resulted in an EtBr-adapted strain, ATCC25923$_{EtBr}$, with reduced susceptibility not only to EtBr, but also to fluoroquinolones, biocides and other dyes. A genotypic

characterization of ATCC25923$_{EtBr}$ revealed that its reduced susceptibility profile was due to a 35-fold increase in the expression level of the efflux pump gene *norA* relatively to the parental strain [7]. This overexpression was, by then, associated with a 70 bp deletion found immediately downstream of the -10 consensus sequence of the *norA* promoter. Thus, that earlier study showed that *S. aureus* can cope with chemical stress by expelling the noxious compound through efflux pumps and that the use of this mechanism confers to the cell a MDR phenotype. We now complemented those earlier observations and demonstrated that the withdrawal of the EtBr stimuli promotes a reversion of the MDR phenotype, rendering the cell even more susceptible to fluoroquinolones, biocides and dyes than the parental strain. This reversion of the MDR phenotype was linked to a reduction of *norA* expression levels, which was accompanied by the loss of the cells overall efflux capacity.

Additional work with strain ATCC25923 showed that the challenge of this strain with a single exposure to a subinhibitory concentration of EtBr (½ the MIC), resulted in the overexpression of not only *norA* (4.51 ± 0.77), but also of all the other chromosomal efflux pump genes tested, namely *norB* (7.07 ± 2.78), *norC* (5.89 ± 0.71), *mepA* (3.90 ± 0.13) and *mdeA* (3.96 ± 2.10) [5].

A first interpretation of these data suggests that the response of *S. aureus* to chemical stress varies according to the duration and the concentration of the stimuli. Overall, a short-term exposure to a sub-inhibitory concentration of EtBr promotes an efflux-mediated response, with the cell activating all efflux systems available (Chapter 5 of this Thesis), whereas a long-term exposure to increasing lethal concentrations of this same compound will, in turn, induce a specific efflux-mediated response, with activation of a single efflux pump, NorA [5, 7]. Additionally, in a more recent study, when ATCC25923 was challenged with a long-term exposure to a constant sub-inhibitory concentration (½ the MIC) or inhibitory (MIC) of EtBr, a third type of response was observed, with overexpression of the *mepA* efflux pump gene (Chapter 7). These findings indicate that *S. aureus* relies on several efflux pumps, not only NorA, to cope with stress stimuli and that it can respond with a multitude of efflux pumps to the same stimulus, as already suggested by other authors [14]. Altogether, these results show that NorA plays a relevant role in long-term responses to chemical stress. In addition, a preferential activation of NorA may be dependent of the concentration of the

drug, with a threshold concentration above which *S. aureus* "switches-off" other efflux systems and maintains NorA active.

2.1.4.2. Factors affecting the expression of *norA*

The deletion found in the ATCC25923$_{EtBr}$ *norA* promoter region, which includes the loss of the majority of the 5'UTR sequence may have a profound effect on the transcription rate of the gene or mRNA stability. The *norA* mRNA of ATCC25923$_{EtBr}$ was found to be slightly less stable than the mRNA of the parental strain. Nevertheless, the reduction in mRNA half-life was not significant and its effect is probably compensated by a higher abundance of the *norA* message in the EtBr-adapted strain. Yet, the deletion may have other effects, namely the creation of a more efficient transcription initiation site or a spatial rearrangement of the *norA* promoter and consequently the hinder of the binding sites of MgrA, making its binding to the promoter less efficient, and thus allowing the transcription of *norA*. These hypothesis were not assayed, but imply an increased transcription of *norA* for both ATCC25923$_{EtBr}$ and ATCC25923$_{EtBr_rev}$. However, a decrease in the expression level was observed for the reverted strain, suggesting alternatively the existence of a post-transcriptional mechanism that regulates *norA*, as has been observed for the regulation of porin expression in *E. coli* [24]. The data obtained may also be interpreted as a combined effect of the 70 bp deletion on *norA* promoter and MgrA overexpression. The effect of MgrA upon the regulation of the *norA* gene is not fully understood. A model has been proposed in which the effect of MgrA is dependent on the cellular ratio of its phosphorylated (MgrA-P) and non-phosphorylated forms [23]. According to this model, MgrA functions as a repressor of the *norA* gene, and upon its phosphorylation, MgrA-P is released from the promoter, allowing *norA* transcription (see also 1.4.3.1.).

In our work, the overexpression of *norA* in ATCC25923$_{EtBr}$ was accompanied by the overexpression of the *mgrA* gene. Additionally, the withdrawal of the EtBr stimulus reduced the expression levels for both genes, with ATCC25923$_{EtBr_rev}$ *mgrA* showing basal expression (0.74 ± 0.51) and *norA* still being overexpressed, but at nearly half (11.89 ± 2.88) the value found for ATCC25923$_{EtBr}$ (34.38 ± 3.36). A first reading of

these results suggests that MgrA is an activator of *norA* expression, contradicting the current model of MgrA modulation. However, the stress induced by the EtBr exposure could have triggered a shift in the balance of the cellular ratio of phosphorylated and non-phosphorylated MgrA towards an increase of MgrA-P proteins. In these conditions, an increase in *mgrA* expression would lead to an increased number of MgrA proteins available for phosphorylation. These phosphorylated MgrA proteins would then be released from or not bind to the *norA* promoter region, allowing the expression of *norA*. According to this hypothesis, the *norA* transcription rate in the EtBr-adapted strain would result from a combined increased transcription due to the 70 bp deletion and a reduced repressor effect of MgrA. Following this premise, the *norA* expression level found in the reverted strain would be solely due to the deletion in the promoter, as the cellular ration of MgrA/MgrA-P would return to its original balance. Additional experiments would be required to clarify these hypothesis, such as the quantification of phosphorylated MgrA and its binding to the *norA* promoter regions for the three strains in study.

2.1.4.3. Mutations affecting NorA activity

The analysis of the ATCC25923$_{EtBr}$ NorA revealed the occurrence of a mutation at the residue 303 replacing a phenylalanine by a tyrosine. This residue is located in the N-terminal of the TMS10. Mutagenesis and site-directed modification studies in the tetracycline MFS transporter TetA(B) showed that the residue in this position was not functionally or structurally important, although a mutation in the neighbour residue affected the transporter activity [21]. The N-terminal of TMS10 is highly hydrophobic and the topology model for this transporter places this segment faced away from the water-filled channel formed by the transporter. Moreover, both phenylalanine and tyrosine are amino acids with similar hydrophobic side chains indicating that the alteration may not alter significantly the spatial arrangement of this transmembrane segment. Thus, it is not expected that this mutation will have a significant effect on ATCC25923$_{EtBr}$ NorA activity. Nevertheless, the analysis of this mutation in a defined genetic system by site-directed mutagenesis should clarify this premise.

Surprisingly, the NorA of the reverted strain ATCC25923$_{EtBr_rev}$ revealed the alteration Tyr-303→Phe and the introduction of a codon stop at position 274. The relieve of the chemical stress imposed to *S. aureus* by the exposure to EtBr lead to a decrease of the expression level of *norA*. However, the maintenance of a relatively high expression level of *norA* (11.89 ± 2.88), and thus of NorA in conditions that no longer required it, could be deleterious for the cell. In fact, some studies have shown that an excess of efflux proteins may have a noxious effect on bacteria [1, 15]. For this reason, a sequence of events in which first occurred the reversion of the Phe-303→Tyr, and secondly the introduction of the codon stop, may have taken place in order to compensate for the increased transcription rate of the *norA* gene in this strain. Yet again, mutagenesis studies should provide information on the transport capacity of the truncated NorA.

2.1.5. Conclusions

The work presented in this sub-Chapter illustrates the relevance of NorA in the response of *S. aureus* to chemical stress, in this case the one imposed by the prolonged exposure to increasing concentrations of EtBr. The overexpression of NorA was directly correlated to a reduced susceptibility profile to a wide variety of antimicrobial compounds, including fluoroquinolones, quaternary ammonium compounds and dyes. The presence/withdrawal of the chemical/EtBr stimulus resulted in an increase/decrease of the *norA* expression levels, and more importantly, exemplified how intricate the regulation of this key efflux system can be.

Recent studies by our group and others, have shown that *S. aureus* does not respond to chemical stress with a preferred efflux pump, but rather through an interplay of the several efflux systems available [5, 14, 16]. Notwithstanding, this work demonstrates the essential role of NorA in protecting *S. aureus* from stress stimuli, with the capacity to render the bacteria with a multidrug resistance phenotype. The recent report that the *norA* gene is also iron responsive, being involved in the export of siderophores [9], reveals that our knowledge of these systems is still scarce concerning substrates other than antimicrobial compounds that can have a high impact on the

resistance profile of this bacterium, as well as the regulatory pathways that modulate the expression of such systems.

2.1.6. References

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2.2. The genetic diversity of the *norA* gene

2.2.1. Introduction

The *norA* gene was first identified in the chromosome of a *S. aureus* fluoroquinolone-resistant isolate collected at a Japanese hospital [20], and found to encode the MFS efflux pump NorA (see 1.4.1.1.) [22]. Some studies have reported the occurrence of some genetic variability within the *norA* gene, and three alleles have been described so far, *norAI* [22], *norA1199* [10] and *norAII* (formerly *norA23*) [15], that differ up to 10% in the nucleotide sequence. Consequently, some reports referred to an absence of *norA* in some *S. aureus* clinical isolates [14, 21], probably due to the failure of the primers used in *norA* screening. This created some confusion in literature that was only solved with the recognition that *norA* is a naturally occurring gene in *S. aureus* showing some degree of genetic variability.

The impact of this genetic diversity on NorA activity remains unclear. In this work, we studied the *norA* alleles present in a collection of ciprofloxacin-resistant *S. aureus* clinical isolates, evaluated their level of expression and their correlation with the respective resistance phenotypes.

2.2.2. Material and Methods

Bacterial strains. The study collection comprised a group of eight isolates (SM1, SM3, SM6, SM10, SM14, SM22, SM50, SM52) representative of a collection of 52 ciprofloxacin-resistant *S. aureus* isolates collected at a Lisbon Hospital, Portugal, during a four-month period [2]. Each strain showed a distintct PFGE type or sub-type. Previous characterization of the efflux capacity of this collection revealed that, with the exception of isolates SM3 and SM6, all presented increased efflux activity associated with reduced susceptibility to fluoroquinolones, biocides and dyes (Chapters 5 and 6) [2, 3]. Strain SM52 also carries the plasmid-encoded efflux pump gene *smr*, which confers reduced susceptibility to biocides and dyes (Chapter 4) [3]. The fully-susceptible

reference strain *S. aureus* ATCC25923 was also studied. All strains were grown in tryptone soya broth (TSB, Oxoid Ltd., Basingstoke, UK) or tryptone soya agar (TSA, Oxoid) at 37ºC. For determination of MICs, strains were cultured in Mueller-Hinton broth (MHB, Oxoid).

Chemical compounds. Antibiotics, biocides and dyes were acquired in powder form from several sources, as follows: EtBr, tetraphenylphosphonium bromide, dequalinium chloride, pentamidine isothionate salt, benzalkonium chloride, berberine, acriflavine, ciprofloxacin (CIP), norfloxacin, levofloxacin, sparfloxacin, nalidixic acid (Sigma, St. Louis, MO, USA), chlorhexidine diacetate, hexadecyltrimethylammonium bromide (CTAB) (Fluka Chemie GmbH, Buchs, Switzerland). All solutions were prepared in the day of the experiment in desionized water.

Drug susceptibility testing. Susceptibility profiles towards antibiotics, biocides and dyes were established by determination of MICs by the broth microdilution method. The MICs of antibiotics were evaluated according to the CLSI guidelines [1]. Cultures were grown overnight in TSB at 37ºC and diluted in PBS to adjust the turbidity to a 0.5 McFarland standard. An inoculum of 0.02 mL of the cellular suspension was then used to inoculate each well of a 96-well plate containing two-fold dilutions of each antimicrobial agent to be tested in MHB. The plates were then incubated at 37ºC for 18 h and the MIC values, corresponding to the lowest concentration of compound that inhibited visible growth, were recorded. All assays were performed in triplicate.

Chromosomal DNA isolation. Genomic DNA was extracted with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), with an additional step of 30 min digestion with lysostaphin (200 mg/L, Sigma) prior to extraction.

Sequence analysis of the *norA* alleles. In order to analyse the circulating *norA* alleles, a 1.4 kb fragment enclosing the *norA* structural gene and its promoter region was sequenced using the conditions described in sub-Chapter 2.1. For PCR amplification and sequencing the following pairs of primers were used: NorA1_fw, 5'-TGTTAAGTCTTGGTCATCTGCA, and NorA1_rv, 5'-CCATAAATCCACCAATCCC; NorA2_fw, 5'- TTCACCAAGCCATCAAAAAG, and NorA2_rv, 5'-CTTGCCTTTCTCCAGCAATA; NorA3_fw, 5'-GGTCATTATTATATTCAGTTGTTG, and NorA3_rv, 5'-

GTAAGAAAAACGATGCTAAT [16]. The pair of primers NorA1_fw/rv and NorA2_fw/rv were designed with the aid of the free-software Primer3 (http://primer3.sourceforge.net/) and tested *in silico* (http://insilico.ehu.es/). Multiple alignments of the sequences were made with the MAFFT software version 6 and edited with the free software BioEdit v. 7.0.9.0 (www.mbio.ncsu.edu/BioEdit/bioedit.html). The phylogenetic tree was elaborated with the software MEGA 5.2 [18], using the method Neighbor-Joining and a matrix of genetic distances calculated by the Kimura with two parameters algorithm (K-2P) [11] with a bootstrap of 1000.

Expression analysis of *norA* alleles. The expression level of the *norA* alleles was analyzed by comparison of the level of *norA* expression in the presence of ciprofloxacin or ethidium bromide, at ½ the MIC for each strain, relatively to: 1) the *norA* expression level in the absence of the compound; 2) the expression level of a wild-type allele. Total RNA was extracted using the RNeasy Mini Kit (QIAGEN) and contaminating DNA was removed with DNase I RNase-free (QIAGEN). RT-qPCR assays were performed using the QuantiTect SYBR Green RT-PCR Kit (QIAGEN) in a Rotor-Gene 3000™ thermocycler with real-time analysis software (Corbett Research, Sydney, Australia). Negative controls and genomic DNA contamination controls were included. 16S rDNA was used as endogenous control. The primers used for the RT-qPCR assays were previously described [2, 4]. The relative quantity of mRNA from each gene was determined by the comparative threshold cycle (C_T) method [13].

2.2.3. Results and discussion

2.2.3.1. Genetic variability among the circulating *norA* alleles

We analyzed the *norA* alleles present in a group of eight clinical strains representative of a collection of 52 ciprofloxacin-resistant isolates, belonging to distinct PFGE types and sub-types [2] and in the susceptible reference strain ATCC25923 (Table 2.3). The entire *norA* coding region and promoter region were sequenced for each strain. Two different alleles of the *norA* gene were found among the selected

isolates, corresponding to the *norAI* and *norAII* alleles previously described, differing up to 10% in the nucleotide sequence and 5% in the polypeptide sequence, reflecting the data for *norA* sequences deposited in public databases (Figure 2.5).

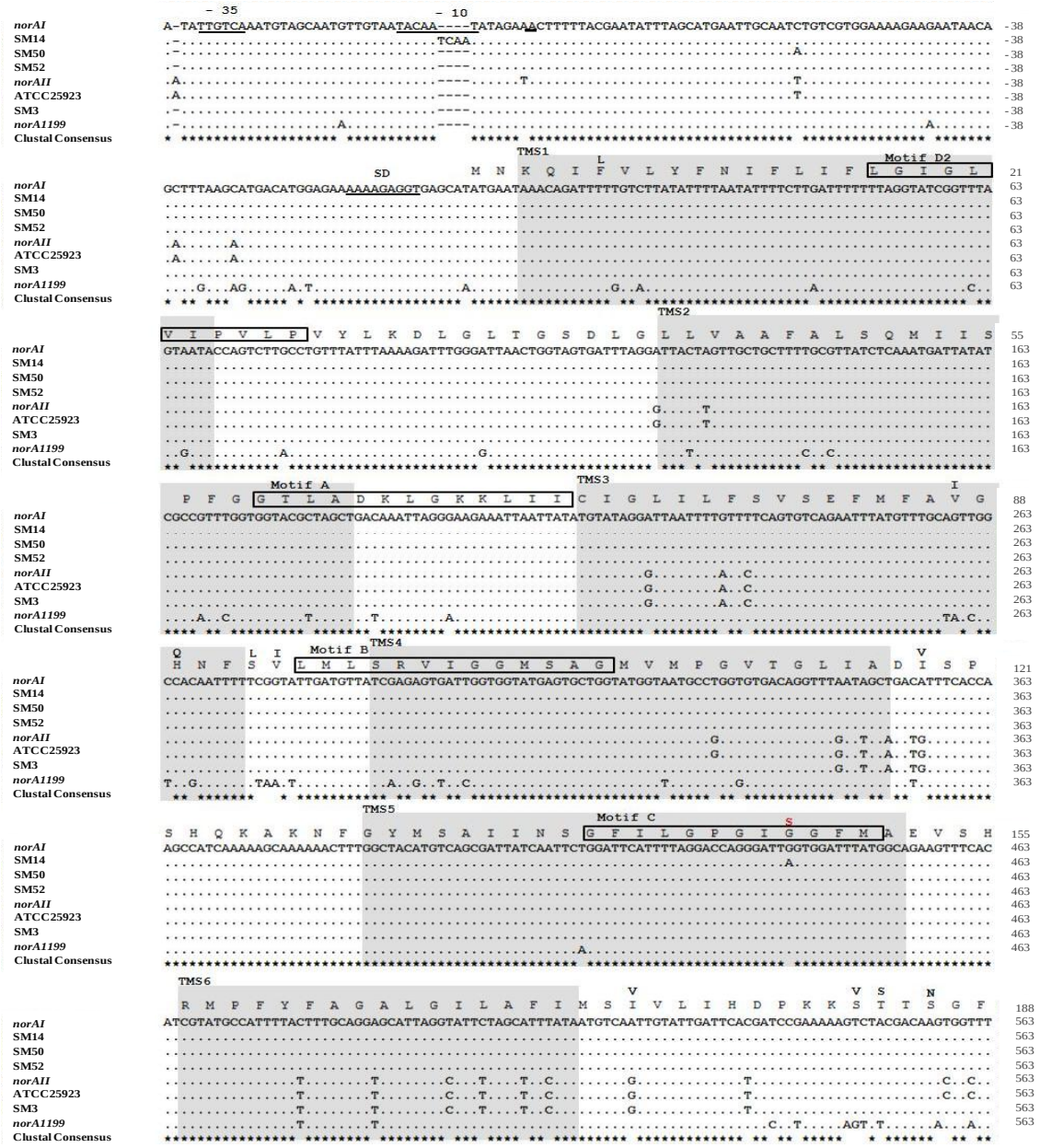

Figure 2.5. Multiple alignment of *norA* sequences from *S. aureus* clinical isolates (SM), ATCC25923 and *norA* sequences described by Yoshida *et al.* (*norAI*), Noguchi *et al.* (*norAII*) and Kaatz *et al.* (*norA1199*). The *norA* sequence of SM1, SM10 and SM22 are identical to the one of SM14. The *norA* of SM6 is identical to *norAI*. The consensus promoter sequences -35, -10, Shine-Dalgarno (SD) and the transcription initiation site of the three *norA* alleles described in literature are underlined in black. The shaded regions correspond to the NorA TMS. The amino acid regions inside the boxes are relative to the conserved motifs described for MFS transporters with 12 TMS. The black residues shown above the main polipeptide sequence represent the variations present in the *norAII*, ATCC25923, SM3 and *norA1199* sequences relative to the *norAI* allele. The red residues represent the mutations found in the clinical isolates.

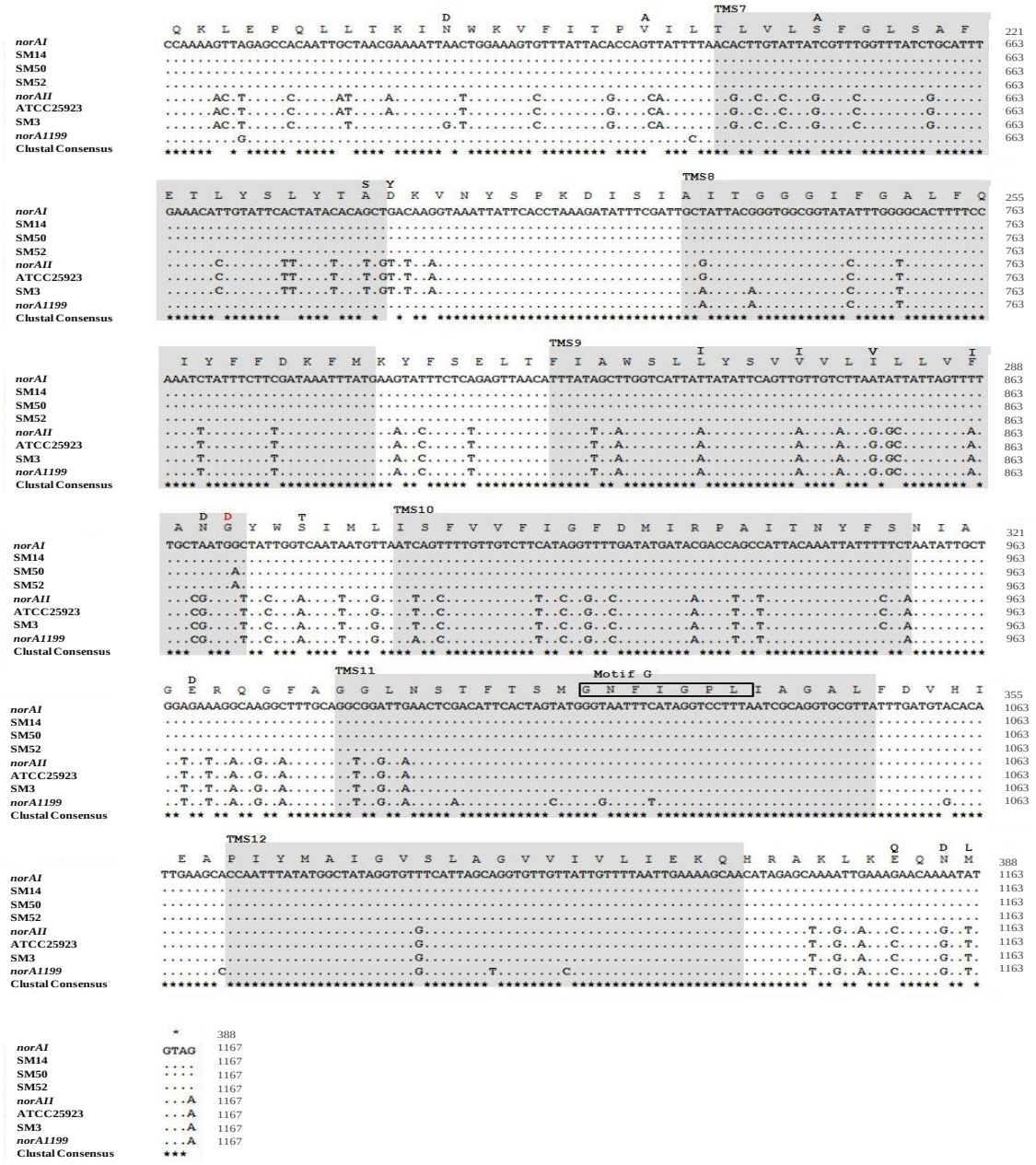

Figure 2.5. ***(Cont.)*** **Multiple alignment of *norA* sequences from *S. aureus* clinical isolates (SM), ATCC25923 and *norA* sequences described by Yoshida *et al.* (*norAI*), Noguchi *et al.* (*norAII*) and Kaatz *et al.* (*norA1199*).**

A phylogenetic analysis of the *norA* alleles found in the collection under study and the sequences from complete *S. aureus* genomes retrieved from public databases was performed and showed a relative conservation of the *norA* gene among all the sequences studied (Figure 2.6). Nevertheless, a topological organization of the *norA* sequences in two clusters could be observed, although these two clusters divide at 74%, which is just below the cut-off value for statistical significance (>75%) [6, 8].

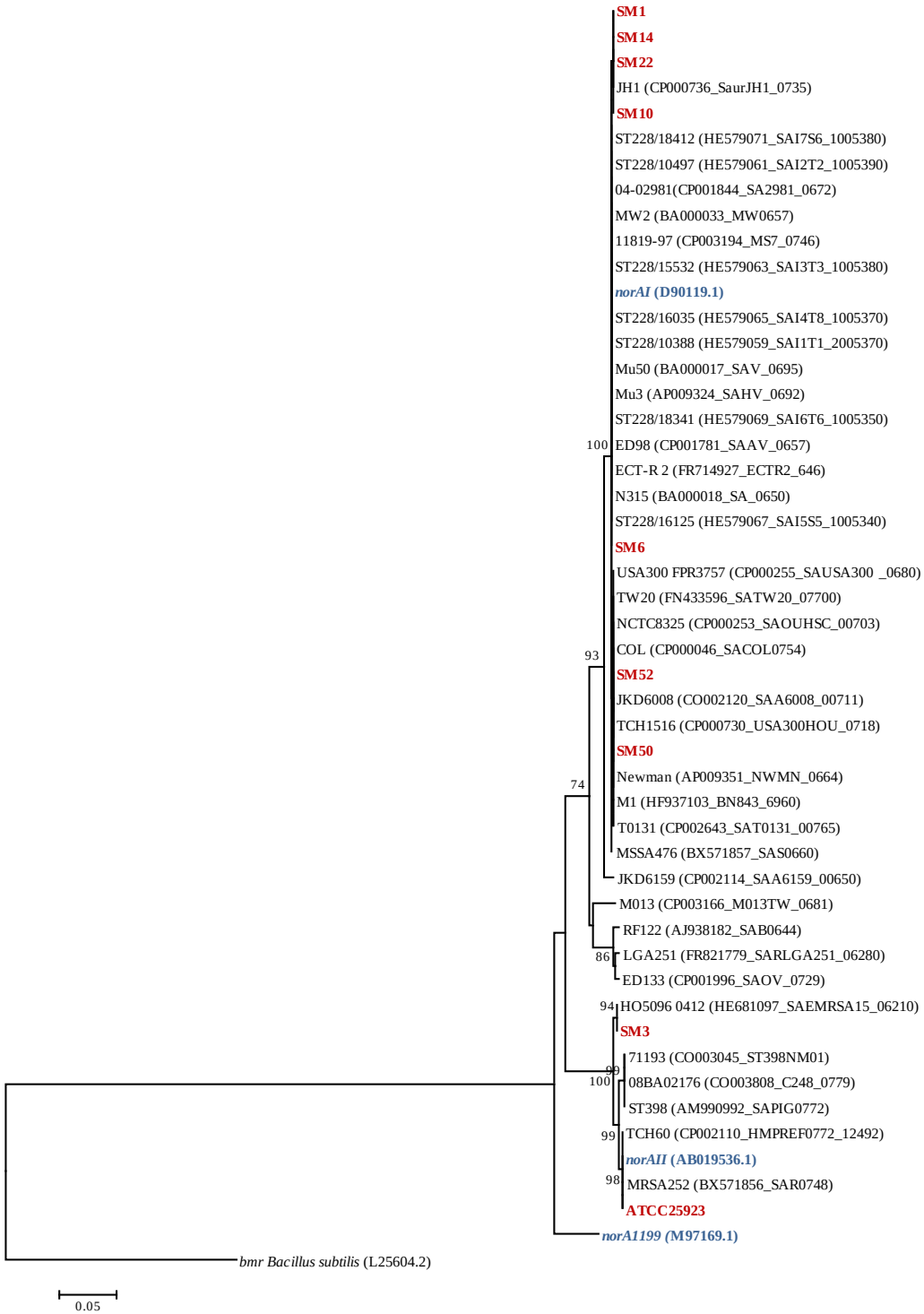

Figure 2.6. Phylogenetic analysis of the *norA* sequences obtained from a representative sub-set of the *S. aureus* ciprofloxacin-resistant clinical isolates (in red), from the complete genome sequences available at public databases (in black) and the three *norA* alleles previously described (in blue). The accession number and locus_tag of each *norA* sequence are presented in brackets after the name of the respective sequence. The phylogenetic tree was elaborated with MEGA 5.2 software using the Neighbor-Joining method and a matrix of genetic distances calculated by the Kimura with two parameters algorithm, with a bootstrap of 1000. The nucleotide sequence from the Bmr multidrug transporter of *Bacillus subtilis* was used as outgroup. A division in clusters is considered statistically significant when >75%.

One cluster includes most of the known sequences as well as the *norAI* allele described by Yoshida *et al.* and all *norA* sequences from clinical strains with PFGE types A, B and C; while the second cluster comprises the *norAII* allele as well as the *norA* sequences of strains SM3 (PFGE type E) and ATCC25923 (Figure 2.6). The position of the *norA1199* allele within the phylogenetic tree is not well defined. Therefore, no conclusion can be made if *norA1199* represents indeed a third allele of *norA*, since the variations found in its sequence can also result from early sequencing errors that may reflect on sequence analysis.

As observed in previous studies [15-17] and as suggested by the phylogenetic tree, *norAI* is the more frequent allele among clinical strains, being present in seven out of the eight strains screened. In our study, the *norAII* allele was detected in a single clinical strain and in the reference strain ATCC25923. Remarkably, several alterations were detected within each *norA* allele (Table 2.3). Among the seven strains carrying *norAI*, four out of the five clone A strains possessed a TCAA insertion immediately downstream of the -10 motif of the promoter region, an event that has been associated with a possible constitutive increased level of *norA* expression [5]. The same strains presented a transition G→A at nucleotide 439 of the NorA coding sequence, which originated the alteration Gly-147→ Ser. The residue 147 is positioned within the motif C (gxxxGPxxGGxl) that is located in TMS5. This motif is conserved among MFS drug:H^+ antiporters, and according to the membrane topology model constructed for the tetracycline TetA(B) MFS transporter, it faces a water-filled channel and may be implicated in the binding of the transporter substrate or proton translocation, functions for which the glycines are thought to be essential [9, 19]. In fact, in a mutagenesis study for the *S. aureus* TetA(K) MFS transporter, an equivalent mutation was responsible for the loss of 80% of the wild-type activity of that efflux pump [7]. Although Tet transporters and NorA are clearly distinct in their substrate specificity, these pumps share the common characteristic of having abundant glycines in TMS5, a trait that has been postulated to confer conformational plasticity to the pumps [7]. Thus, the mutation detected at the *norA* structural gene may be implicated in a reduction of NorA activity.

The *norAI* allele of strain SM6 (PFGE type A) revealed to be identical to the wild-type allele. The remaining two strains carrying *norAI*, SM50 and SM52 (with PFGE types B and C), possessed a transition G→A in *norA* nucleotide 872, which was

responsible for the replacement Gly-291→Asp (Table 2.3). According to the membrane topology model of TetA(B), this residue is located in the loop between TMS9 and TMS10, and was shown not to be relevant for the function of this transporter [12, 19]. This NorA mutation has already been described in *S. aureus* clinical isolates, but no correlation with the susceptibility phenotype could be retrieved since it occurred in either fluoroquinolone-resistant or -susceptible isolates [16]. Strain SM50 also showed a mutation in the *norA* promoter region that may alter *norA* expression (Table 2.3).

The two strains carrying *norAII*, the reference strain ATCC25923 and SM3 (PFGE type E), evidenced a higher number of variations in the *norA* sequence (Figure 2.6 and Table 2.3). Most of these variations corresponded to silent mutations. In addition, SM3 *norA* presented a transition A → G in nucleotide 600, resulting in a substitution Asn-200→Asp. According to the membrane topology model of TetA(B), this residue is situated in the central loop, between TMS6 and TMS7, but with no essential role assigned [19]. Strain SM3 also showed a deletion of a nucleotide in the *norA* promoter region, which could affect the expression of the gene.

Table 2.3. Characterization of the promoter and structural regions of the *norA* alleles of *S. aureus* clinical isolates in study.

Isolate	PFGE pattern	*norA* allele	Variations to the original *norA* allele	
			***norA* promoter** [1]	**Structural gene** [2]
SM1	A2	***norAI***	Ins TCAA (-104)	G → A (439) Gly-147→Ser
SM10	A4		Ins TCAA (-104)	G → A (439) Gly-147→Ser
SM14	A3		Ins TCAA (-104)	G → A (439) Gly-147→Ser
SM22	A1		Ins TCAA (-104)	G → A (439) Gly-147→Ser
SM50	B1		C → A (-61)	G → A (872) Gly-291→Asp
SM52	C1		no alterations	G → A (872) Gly-291→Asp
SM6	A5		no alterations	no alterations
ATCC25923	---	***norAII***	T → A (-94)	no alterations
SM3	E1		Del A (-131)	A → G (600) Asn-200→Asp several silent mutations

Ins: insertion; Del: deletion; Gly: glycine; Ser: serine; Asp: aspartic acid; Asn: asparagine. [1]The region analyzed encloses the nucleotides -232 to -1. [2]The region analyzed encloses the entirety of the *norA* nucleotide sequence, from nucleotides 1 to 1167.

2.2.3.2. Analysis of the expression level of the *norA* alleles

The level of expression of the two *norA* alleles detected among the clinical strains was analyzed by three approaches. First, *norA* expression was evaluated after exposure of the strains to a sub-inhibitory concentration of two known NorA substrates, ciprofloxacin and ethidium bromide. The results obtained revealed no increased expression of both *norAI* and *norAII* in the presence of either compound (Table 2.4). Only the reference strain ATCC25923, carrying the *norAII* allele, showed overexpression in the presence of EtBr. This result suggests that EtBr, in comparison with ciprofloxacin, may be a preferred substrate of NorA for this strain. In the second approach, we looked for possible changes in the *norA* expression of strains sharing very similar genetic backgrounds (same PFGE type). This approach would hinder possible biased results due to differences in the strain background. Thus, the level of expression of *norAI* was assessed in strain SM1, carrying an insertion in the promoter region, in comparison with the *norAI* allele carried by SM6 with no mutations in the promoter, in the presence of ciprofloxacin or EtBr (Table 2.4). In the presence of ciprofloxacin the SM1 *norAI* allele showed a consistent increased expression compared to the SM6 allele, although at a lower level than the one considered for overexpression (Table 2.4). This preliminary result may indicate that ciprofloxacin is a better inducer of *norAI* transcription. These two approaches are good strategies for detection of inducible expression, but unable to provide data on the constitutive/basal level of gene expression. To counteract this fact, the level of *norAI* expression in SM1 was calculated relatively to SM6 in the absence of inducers. This strategy would then report on the potential increased expression level of the SM1 *norAI* allele due to the presence of the insertion in the promoter. However, the data obtained revealed again no overexpression of the SM1 *norAI* allele, thus implying no relevant role for this promoter alteration in contrary to what has been proposed [5].

Table 2.4. Determination of the level of expression of the circulating *norA* alleles.

Strain	*norA* allele	*norA* expression level [1] (Mean ± SD)				
		vs. drug-free condition		vs. wild-type allele (SM6)		
		CIP	EtBr	CIP	EtBr	No inducer
SM1	***norAI***	1.03 ± 0.59	1.86 ± 1.62	3.25 ± 0.00	1.17 ± 0.65	0.22 ± 0.19
SM50		0.72 ± 0.15	0.96 ± 0.55	---	---	---
SM52		0.61 ± 0.32	0.86 ± 0.66	---	---	---
SM6		0.96 ± 0.91	1.01 ± 0.86	---	---	---
ATCC25923	***norAII***	0.30 ± 0.10	4.51 ± 0.77	---	---	---
SM3		2.03 ± 1.22	2.22 ± 0.81	---	---	---

SD: standard deviation; CIP: ciprofloxacin; EtBr: ethidium bromide; [1]The results are expressed as the mean of at least two independent assays performed with independently extracted total RNAs. Overexpression is only considered when the expression level is ≥4 [2].

2.2.3.3. Relation between *norA* alleles and resistance phenotypes

Despite the early data regarding the genetic diversity of *norA* [15-17], these studies were not able to establish a link among *norA* genetic variability and resistance to antimicrobial compounds and therefore the clinical relevance of *norA* diversity remains unclear.

The susceptibility profile towards known NorA substrates, namely fluoroquinolones, biocides and dyes was determined for all strains in study (Table 2.5). Regarding fluoroquinolones, all strains carried mutations in the quinolone-resistance determining region (QRDR) of the target genes *grlA* and *gyrA* that confer high-level resistance to these antibiotics. Nevertheless, some differences in the fluoroquinolone MICs were detected among the strains. Strains SM1, SM10, SM14 and SM22 carrying NorAI(Gly-147→Ser), presented MICs of ciprofloxacin and norfloxacin two to four-fold higher than the ones for SM50 and SM52, producing NorAI(Gly-291→Asp). This difference was higher, eight to 16-fold, when compared to the MICs for the strains SM6 (wild-type NorAI) and SM3, carrying NorAII. Evaluating the MICs of biocides and dyes, some differences were also detected among the strains. In particular, the MICs of

EtBr, benzalkonium chloride, CTAB, and dequalinium chloride were two to eight-fold higher for the strains carrying NorAI, in comparison with the strains carrying NorAII (Table 2.5). The increased MIC values of some biocides detected for SM52 can be attributable to the presence of the *smr* gene encoding the Smr efflux pump (Chapter 4). Overall, these results are in agreement with a higher efflux activity for NorAI strains. This increased efflux can not be directly associated with NorA activity since the contribution of other efflux pumps is not fully taken into account. Data from other studies and our work (Chapters 5 and 6) show that other *S. aureus* chromosomal efflux pumps may also be implied in the extrusion of some of these compounds and thus, the observed phenotypes can not be directly assigned to each type of NorA or *norA* allele. Assuming no alteration in the activity of other efflux systems, one can compare the data for strains carrying wild-type and altered NorA pumps, particularly for closely-related strains. Accordingly, it can be hypothesized that the NorA pumps carrying the mutation Gly-147 → Ser would have increased activity. This hypothesis is in opposition to the predicted effect of this mutation on NorA. Clarification of this information would require the assay of these altered NorA pumps in defined genetic systems.

In general, the data gathered suggests that, as observed in other studies [15-17], *norAI* allele occurs more frequently. Several alterations were found in both *norA* structural gene and promoter region. In particular, the mutation Gly-147 → Ser was detected in strains carrying *norAI* and despite an expected association with a decreased NorA activity, these strains were the ones presenting the highest MIC values for several of the NorA substrates tested. This result suggests an increase in NorA activity due to this mutation, although the role of the other chromosomal efflux pumps is not being taken in account. No alteration in NorA activity could also be assigned to strains carrying NorA with the Gly-291→Asp or Asn-200→Asp mutations. This represents the main challenge in studying the individual role of *S. aureus* chromosomally-encoded efflux pumps, since other pumps may also contribute to the phenotypes and even modify their expression levels to balance alterations occurring in key pumps, such as NorA. More definitive data on the individual role of NorA variants would require site-mutagenesis assays conducted in defined genetic systems.

Table 2.5. Characterization of the *S. aureus* clinical isolates regarding their *norA* alleles, QRDR mutations and MIC values (mg/L) for several known NorA substrates.

Strain	*norA* allele	QRDR mutations		MIC (mg/L)													
				fluoroquinolones					biocides and dyes								
		GrlA	GyrA	CIP	NOR	LEV	NAL	SPX	EtBr	ACR	BER	CHX	BAC	CTAB	TPP	PT	DQ
SM1	***norAI***	S80Y E84G	S84L	128	512	64	256	16	16	32	512	1	2	8	32	64	4
SM10		S80Y E84G	S84L	128	512	64	128	32	16	32	256	1	2	8	32	64	4
SM14		S80Y E84G	S84L	256	1024	32	256	16	16	32	512	1	4	8	64	64	16
SM22		S80Y E84G	S84L	128	512	32	64	16	16	16	512	1	4	4	64	64	16
SM50		S80F E84K	S84L	64	256	64	128	16	8	32	128	0.5	2	2	32	16	4
SM52		S80Y	S84L	16	64	16	128	16	16	16	>1024	1	2	8	16	16	4
SM6		S80F	S84L	16	64	8	64	8	4	16	128	0.5	1	2	8	16	1
ATCC25923	***norAII***	WT	WT	0.25	0.5	0.125	64	0.125	6.25	16	128	0.5	1	1	16	32	1
SM3		S80F E84G	S84L	16	64	8	64	16	2	16	256	0.5	1	1	16	16	2

CIP: ciprofloxacin; NOR: norfloxacin; LEV: levofloxacin; NAL: nalidixic acid; SPX: sparfloxacin; EtBr: ethidium bromide; ACR: acriflavine; BER: berberine; CHX: chlorhexidine; BAC: benzalkonium chloride; CTAB: hexadecyltrimethylammonium; TPP: tetraphenylphosphonium bromide; PT: pentamidine isothionate salt; DQ: dequalinium chloride; QRDR: quinolones-resistance determining region; S: serine: Y: tyrosine; E: glutamate; G: glycine; L: leucine; F: phenylalanine; K: lysine; WT: wild-type.

Gene expression assays revealed no higher values of expression of both *norA* alleles in the absence or presence of EtBr or ciprofloxacin. These results suggest that the mutations found in the *norA* promoter region of strains SM1, SM3 and SM50 may pose no effect on the expression rate of *norA*, contrary to what has been found in other studies. However, they could also reflect the approaches used, as the basal level of *norA* expression in each strain was used as the initial point for the comparisons of expression levels and thus is not directly measured. Nevertheless, increased *norAI* expression was found for strain SM1, when compared to the SM6 wild-type *norAI* in the presence of ciprofloxacin, but not EtBr. This result may indicate that ciprofloxacin could be a better inducer of *norAI* expression in this strain. Altogether, these data support the need of additional studies with defined genetic systems to fully address the question of the impact of the genetic diversity of *norA* on the expression of the gene and on the resistance to antimicrobials.

2.2.4. Conclusions

Our study has described the occurrence of more than one allele of the *norA* efflux pump gene in a collection of 52 *S. aureus* clinical isolates and that *norAI* is the predominant allele in *S. aureus*, reinforcing previous findings. This type of work is relevant for studies with key proteins and should be further explored. As in earlier studies, a clear association between *norA* alleles and susceptibility profiles to antimicrobial compounds was difficult to establish. Our results also sustain the necessity of conducting mutagenic studies with NorA, in order to gain insights into the actual contribution of this MDR efflux pump to the reduced susceptibility phenotypes presented by *S. aureus*, in particular fluoroquinolones and biocides. These studies are also essential to elucidate the interplay of NorA with other MDR efflux pumps present in *S. aureus*, contributing to a wider perception of the resistance mechanisms in this bacterium. This topic will be further explored in Chapters 5, 6 and 7 of this Thesis.

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CHAPTER 3

The role of QacA in efflux-mediated resistance

3.1. Identification of the plasmid encoded *qacA* efflux pump gene in the methicillin-resistant *Staphylococcus aureus* (MRSA) strain HPV107, a representative of the MRSA Iberian clone

This sub-chapter contains data published in:

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3.2. Role of pSM39, a multiresistance plasmid harboring the *qacA* gene for biocide resistance, on the resistance phenotype of a MRSA strain

Summary

Biocides are pivotal for infection control and prevention, being extensively used in healthcare settings. They are also widely used in animal husbandry, several industries and increasingly in domestic households. Reduced susceptibility to biocides has been reported for several decades in many bacterial species. MRSA is a major nosocomial pathogen, becoming increasingly prevalent also in the community, for which prevention and control measures consist mainly of the application of biocides with antiseptic and disinfectant activity. In this chapter the presence of the efflux pump plasmid-located gene *qacA* was demonstrated in strain HPV107, a clinical isolate representative of the Iberian MRSA clone (sub-Chapter 3.1). The existence of efflux activity in strain HPV107 due to the QacA pump was also established and correlated to a phenotype of reduced susceptibility towards several biocide compounds. No association could be made with antibiotic resistance. It is also presented data on the study of the role of QacA in a MRSA human clinical isolate, SM39, collected at a Portuguese hospital (sub-Chapter 3.2). As in strain HPV107, the QacA efflux activity could be associated with reduced susceptibility to antiseptics and disinfectants in SM39. The genetic context of the *qacA* gene was also explored and a description of a large multiresistance plasmid is presented, belonging to the β-lactamase/heavy-metal family. Accordingly, this plasmid carries additional determinants for resistance to β-lactams and heavy-metals (cadmium and mercury) and sequence analysis indicate that it may be a product of a rearrangement between plasmids with origin in *S. aureus* and the coagulase-negative *Staphylococcus epidermidis*.

These two studies emphasize the potential of the QacA pump activity in the maintenance and dissemination of important MRSA strains in the hospital setting and increasingly in the community.

3.1. Identification of the plasmid encoded *qacA* efflux pump gene in the methicillin-resistant *Staphylococcus aureus* (MRSA) strain HPV107, a representative of the MRSA Iberian clone

3.1.1 Introduction

Treatment, prevention and control of infectious diseases have undergone remarkable changes during the last century. Use of antibiotics to eliminate pathogenic bacteria, combined with the application of chemical compounds for disinfectant and/or antiseptical purposes (also known as biocides) has allowed the management of several pathogenic bacteria known to cause life-threatening conditions [5]. However, the selective pressure derived from the increasing usage of these antimicrobial compounds combined with the adaptability of bacteria has resulted in the emergence of bacterial strains resistant to representatives of one or more antibiotic classes as well as to biocides [5]. Bacteria that possess a multidrug-resistant (MDR) phenotype are of foremost concern in healthcare settings inasmuch as they are problematic for therapy.

Amongst the known mechanisms of resistance to antibiotics, the activity of multidrug efflux pumps is a major contributor to the development of MDR phenotypes [11]. In the pathogenic bacterium *Staphylococcus aureus*, several multidrug efflux pumps have been described and were found to be encoded by chromosomal genes (*norA, norB, norC, mepA, mdeA, sepA* and *sdrM*) as well as genes located on plasmids (e.g. *qacA/B, smr, qacG, qacJ* and *qacH*) [11]. Usually, chromosomally encoded multidrug efflux pumps can confer reduced susceptibility to either antibiotics or biocides, whilst the plasmid-encoded pumps can only confer reduced susceptibility to biocides [11]. Two such examples are the multidrug efflux pump QacA and the closely related QacB that extrude from *S. aureus* a wide variety of monovalent (both QacA and QacB) and divalent cationic compounds (only QacA) using the proton-motive force [1].

This study reports the finding of the multidrug efflux pump QacA in a clinical methicillin-resistant *Staphylococcus aureus* (MRSA) strain HPV107, representative of

the so-called Iberian clone that is currently disseminated throughout several European countries and the USA [4].

3.1.2 Material and Methods

Strains. The strain HPV107, representative of the MRSA Iberian clone, was isolated at a Portuguese hospital in 1992 and is characterized by resistance to several classes of antibiotics, particularly β-lactams, aminoglycosides, fluoroquinolones, macrolides, rifampicin and tetracycline [9, 10, 12]. Strain $HPV107_{cured}$ corresponds to MRSA strain HPV107 cured of plasmids. *S. aureus* ATCC25923, a clinical isolate collected in Seattle, USA, in 1945 and fully susceptible to antibiotics, was used as a reference strain. Strains were grown in tryptone soya broth (TSB) or agar (TSA) (Oxoid Ltd., Basingstoke, UK) at 37^{o}C. For MIC determination, cultures were grown in Mueller-Hinton broth (MH, Oxoid) at 37ºC.

Compounds. All antibiotics, biocides, dyes and efflux inhibitors (EIs) were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Fluka Chemie GmbH (Buchs, Switzerland).

Minimum inhibitory concentration (MIC) determination. MICs of the study strains towards several antibiotics (oxacillin, ciprofloxacin, norfloxacin, levofloxacin, sparfloxacin, gentamicin, trimethoprim, kanamycin, chloramphenicol, tetracycline and erythromycin), biocides (tetraphenylphosphonium bromide, dequalinium chloride, benzalkonium chloride, hexadecyltrimethylammonium bromide, cetrimide, chlorhexidine and pentamidine isothionate salt) and dyes (ethidium bromide, berberine, acriflavine and crystal violet) was determined by the two-fold microdilution broth method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [2], in the absence and presence of EIs, namely thioridazine (TZ), chlorpromazine (CPZ), verapamil (VER) and reserpine (RES).

Plasmid DNA isolation and plasmid curing. Plasmid DNA was isolated using a QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany), with an additional incubation step with lysostaphin (35 mg/L, Sigma) at 37ºC for 90 min prior to cell lysis.

HPV107 was cured of plasmids by successive passage in medium containing increasing subinhibitory concentrations of chlorpromazine (from 2 mg/L to 22 mg/L) [8] or novobiocin (2 µg/L to 125 µg/L) [6]. The efflux capacity of each subculture was evaluated by plating overnight cultures on TSA media containing the broad-range efflux pump substrate ethidium bromide. Cells with lower efflux activity will not be able to extrude the fluorochrome ethidium bromide, substrate which accumulates within the cell producing pink fluorescence under ultraviolet light. On the other hand, cells with higher efflux activity will be able to extrude ethidium bromide, requiring higher ethidium bromide concentrations to fluoresce. Thus, by inoculating each of the subcultures obtained on TSA plates containing 2.5 mg/L ethidium bromide, it was possivle to select non-fluorescent and fluorescent colonies (potentially lacking the plasmid) [6].

Screening of efflux pump genes by PCR and enzymatic restriction analysis. Plasmid-mediated efflux pump genes were screened by PCR, using specific primers for *qacA/B* and *smr* [3]. The reaction mixture (0.05 mL) contained 2.5 U of Taq Polymerase (Fermentas Inc., Ontario, Canada), 1X Taq buffer (Fermentas); 25 pmol of each primer; 0.2 mM of dNTP and 1.75 mM of $MgCl_2$. The PCR reactions were conducted in a thermocycler Mastercycler personal 5332 (Eppendorf AG, Hamburg, Germany), under the following conditions. DNA was denatured at 95ºC for 1 min, followed by 30 cycles of denaturation at 95ºC each for 1 min, annealing at 40ºC (*qacA/B*) or 48ºC (*smr*) and extension at 72ºC for 1 min, followed by a step of final extension at 72ºC for 5 min. Amplification products were visualized by 1% agarose gel electrophoresis. The resulting *qacA/B* fragment was purified and sequenced on both strands with the same set of primers. Sequence aligment and analysis was conducted with the aid of programs ClustalW and BioEdit, version 7.0.8.0, respectively. The *qacA/B* internal fragment was digested with the restriction enzyme AluI (Fermentas) at 37ºC for two hours. Restriction products were analyzed by 3.5% Metaphor agarose gel (Lonza, Rockland, USA) electrophoresis.

Macrorestriction analysis. HPV107 and $HPV107_{cured}$ were typed by pulsed-field gel electrophoresis (PFGE) analysis using well-established protocols. Briefly, agarose disks containing intact chromosomal DNA were prepared as previously described [3] and

were restricted with SmaI according to the manufacturer's recommendations (New England Biolabs, Ipswich, MA, USA). Restriction fragments were then resolved by PFGE, which was carried out in a contour-clamped homogeneous electric field (CHEF) apparatus (CHEF-DRIII, Bio-Rad, Hercules, CA) as previously described [3].

Semi-automated fluorometric method. Efflux activity of the study strains was evaluated using a previously described semi-automated fluorometric method [3, 16]. This method, with the aid of the Rotor-Gene 3000™ thermocycler and real-time analysis software (Corbett Research, Australia), allows real-time detection of accumulation and efflux of the fluorescent efflux pump substrate ethidium bromide.

3.1.3 Results and discussion

As expected, the susceptibility profiles of strain HPV107 and the reference strain *S. aureus* ATCC25923 towards several antibiotics, biocides and dyes showed a fully resistant profile of HPV107 to most of the antibiotics tested together with reduced susceptibility to all biocides compared with the reference strain (Table 3.1). However, in the presence of efflux inhibitors (EIs), in particular thioridazine (TZ), a general decrease in the minimum inhibitory concentration (MIC) for biocides and dyes was observed (Table 3.1). This observation suggests that HPV107 has an efflux activity that correlates with decreased susceptibility to biocides and dyes. Chromosomally encoded efflux pumps usually possess a range of substrates that include not only biocides and dyes, but also antibiotics such as fluoroquinolones. MICs for HPV107 of fluoroquinolones also decreased with some EIs, although to a lesser extent than that observed for the biocides and dyes. Thus, the phenotype presented by HPV107 towards biocides and dyes probably results mainly from a multidrug efflux pump gene carried on a plasmid. To confirm this hypothesis, the presence of the plasmid-located efflux pump genes *qacA/B* and *smr* was screened by PCR in the *S. aureus* study strains. An internal fragment of the *qacA/B* gene was amplified by PCR (data not shown). To distinguish between the genes *qacA* and *qacB*, which only differ by seven nucleotides,

the *qacA/B* internal fragment was digested with the restriction enzyme AluI, which recognises an additional restriction site within *qacB* [13].

Table 3.1. Characterization of the *S. aureus* strain ATCC25923, MRSA strain HPV107, and HPV107$_{cured}$ susceptibility profiles against antibiotics, biocides and dyes in the absence and presence of ½ the MIC of efflux inhibitors (EIs).

Antimicrobial compound			MIC (mg/L)[a]					
			ATCC25923	HPV107[b]				HPV107$_{cured}$
				No EI	+ TZ	+ CPZ	+ VER	+ RES	
Antibiotics		CIP	0.25 (S)	16 (R)	8	8	16	16	16 (R)
		NOR	0.5 (S)	64 (R)	32	32	**16**	32	64 (R)
		LEV	0.125 (S)	8 (R)	8	8	8	8	8 (R)
		SPX	0.125 (S)	16 (R)	16	16	8	8	16 (R)
		OXA	0.125 (S)	128 (R)	64	64	128	128	128 (R)
		TET	0.25 (S)	32 (R)	16	16	16	32	16 (R)
		ERY	0.5 (S)	>1024 (R)	1024	1024	>1024	>1024	>1024 (R)
Biocides and dyes	Monovalent	TPP	12.5	256	**8**	**32**	**64**	256	8
		BAC	0.75	4	**1**	2	2	4	1
		CTAB	0.75	8	**2**	4	4	4	2
		EtBr	6.25	256	**32**	**64**	**64**	128	1
		BER	200	>1024	1024	>1024	>1024	>1024	128
		ACR	16	>512	512	>512	>512	>512	16
		CV	0.03	1	**0.125**	**0.25**	0.5	1	0.125
	Divalent	DQ	6.25	32	**0.5**	**2**	**1**	16	4
		CHX	0.375	1	0.5	0.5	0.5	1	1
		PT	50	256	**32**	128	128	256	16

EI: efflux inhibitor; TZ: tioridazine, CPZ: chlorpromazine; VER: verapamil; RES: reserpine; CIP: ciprofloxacin; NOR: norfloxacin; LEV: levofloxacin; SPX: sparfloxacin; OXA: oxacillin; TET: tetracycline; ERY: erythromycin; TPP: tetraphenylphosphonium bromide; BAC: Benzalkonium chloride; CTAB: hexadecyltrimethylammonium bromide; EtBr: ethidium bromide; BER: berberine; ACR: acriflavine; CV: crystal violet; DQ: dequalinium chloride; CHX: chlorhexidine; PT: pentamidine isothionate salt. [a] Letters in parenthesis correspond to the phenotype of the isolate according to the CLSI guidelines, S – susceptible and R – resistant. Values in bold correspond to a decrease in the original MIC by ≥ 4X in the presence of an EI. [b] The following EI concentrations were used: 6.25 mg/L of TZ; 12.5 mg/L of CPZ; 200 mg/L of VER; 20 mg/L of RES.

Thus, when the *qacA* amplification product is restricted with AluI, several fragments are produced, the larger of which has 200 bp, whilst those obtained from the

qacB gene are of a significantly smaller size. Since a 200-bp AluI fragment was detected (Figure 3.1-A), we could infer that the plasmid present in HPV107 carries the *qacA* gene and not the *qacB*. Sequencing the *qacA/B* fragment corroborated the identification of the *qacA* gene. These conclusions were supported by the decrease in MIC values determined for divalent cationic biocides in the presence of EIs, which are substrates for QacA only (Table 3.1).

To confirm the location of the *qacA* gene in HPV107, the strain was cured of plasmids by exposure to chlorpromazine or novobiocin. The resulting cultures were then screened for the presence or absence of plasmids using ethidium bromide as an indicator of efflux activity. The resulting fluorescent colonies presented morphology similar to the non-fluorescent colonies, albeit slightly smaller. Plasmid DNA extraction was carried out from both fluorescent and non-fluorescent colonies. No plasmids could be obtained from the fluorescent colonies in contrast to the non-fluorescent colonies that were found to carry a single 32-kb plasmid. These results indicated that the *qacA* gene was carried on a 32-kb plasmid in strain HPV107 and were supported by PCR amplification for the *qacA/B* internal fragment, which was positive for the non-fluorescent colonies and negative for the fluorescent colonies (Figure 3.1-B). One of the fluorescent colonies obtained after successive passage with chlorpromazine was selected for further testing and was designated $HPV107_{cured}$. PFGE (pulsed-field gel electrophoresis) patterns showed that no contamination occurred during the plasmid curing procedure (data not shown). A single difference was detected among the PFGE patterns of HPV107 and $HPV107_{cured}$, which corresponded to a fragment of ca. 30 kb, which was detectable for HPV107 only and may correspond to the *qacA*-carrying plasmid. MICs of $HPV107_{cured}$ for the antibiotics, biocides and dyes previously tested were determined and it was found that $HPV107_{cured}$ maintained the antibiotic-resistant profile, but it became more susceptible to the biocides and dyes tested, with the exception of chlorhexidine (Table 3.1). Analysis of other fluorescent and non-fluorescent colonies revealed similar phenotypes.

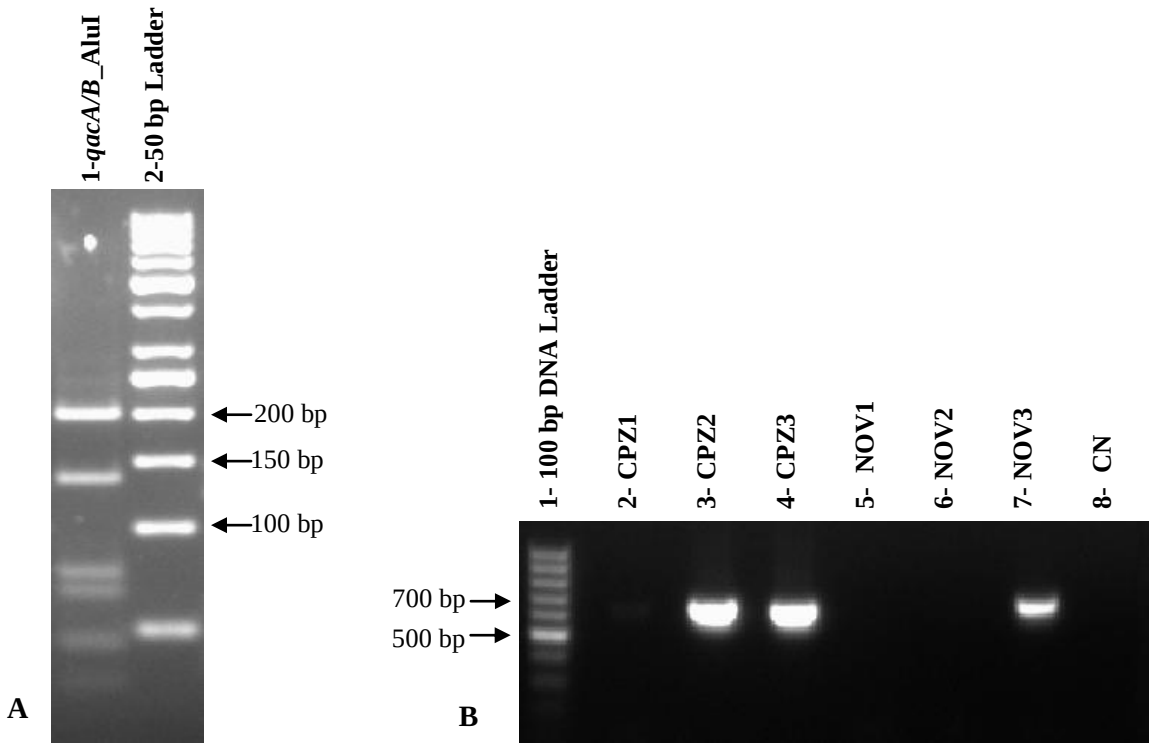

Figure 3.1. (A) Restriction products of the *qacA/B* internal fragment digested with AluI, resolved in 3.5% Metaphor agarose gel (B) Amplification products with *qacA/B* primers (628 bp) from colonies selected after plasmid curing with either chlorpromazine (CPZ): 2: CPZ1, fluorescent colony; **3:** CPZ2, non-fluorescent colony; **4:** CPZ3, non-fluorescent colony; **or novobiocin (NOV): 5:** NOV1, fluorescent colony; **6:** NOV2: fluorescent colony; **7:** NOV3: non-fluorescent colony; **1:** DNA Ladder 100 bp Gene Ruler (Fermentas); **8:** CN, negative control for *qacA/B* amplification.

To confirm further that the activity of the QacA pump accounted for the reduced susceptibility to a diverse panel of biocides and dyes in HPV107, the efflux activities of HPV107, $HPV107_{cured}$, and *S. aureus* ATCC25923 were characterized by a semi-automated fluorometric method as previously described [3, 16]. Data obtained showed that HPV107 had a higher efflux activity in comparison with that presented by *S. aureus* ATCC25923 (Figure 3.2-B) and, consequently, a lower retention of ethidium bromide (Figure 3.2-A).

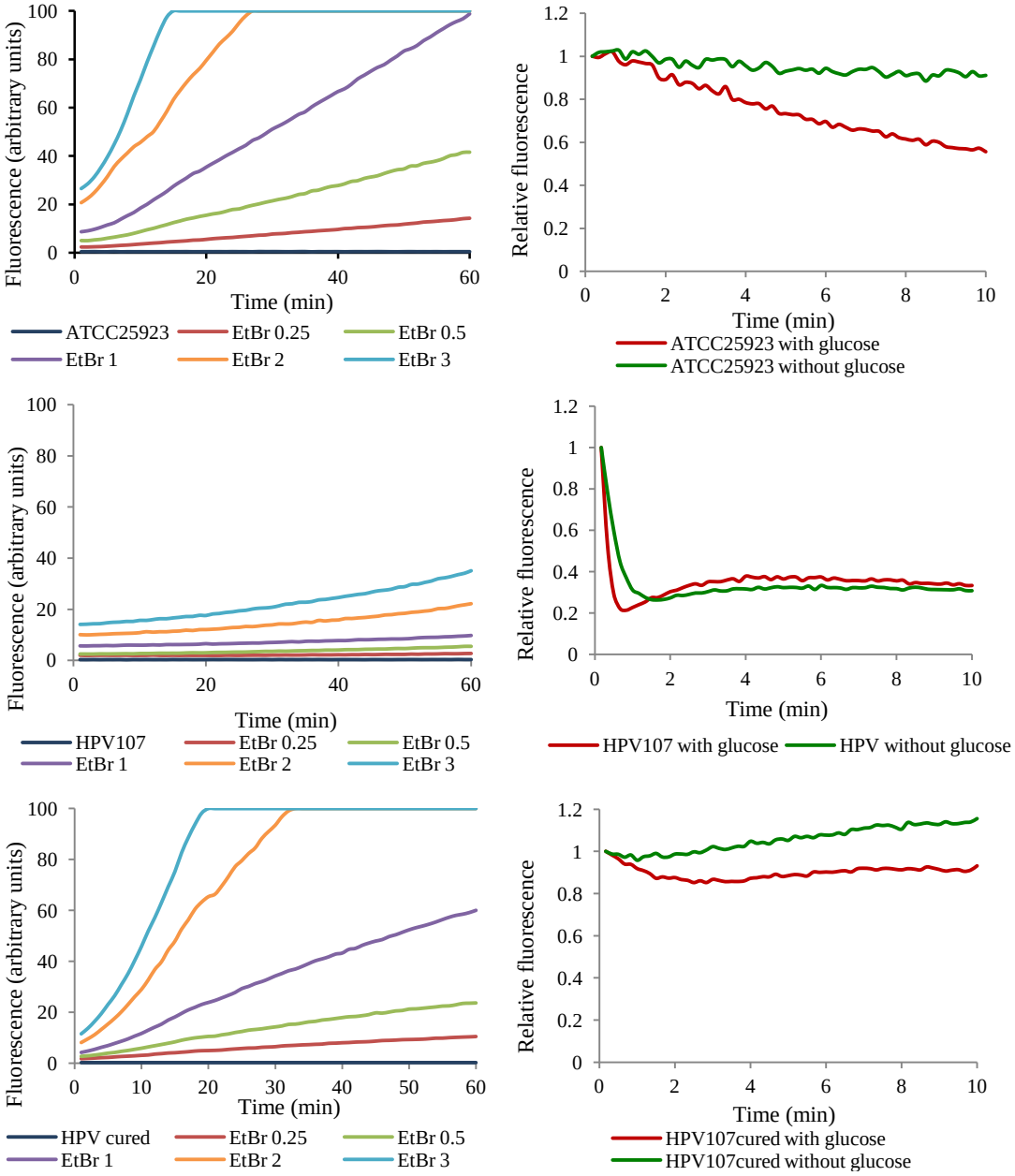

Figure 3.2. Analysis of ethidium bromide (EtBr) accumulation and efflux by a semi-automated fluorometric method. (A) Accumulation assays of the strains ATCC25923, HPV107 and HPV107$_{cured}$ in the presence of increasing EtBr concentrations (mg/L). **(B)** Efflux assays of the strains ATCC25923, HPV107 and HPV107$_{cured}$ in the presence of glucose 0.4% (p/v), washed after a period of incubation of 60 minutes at 25ºC in the presence of EtBr at 0.25 mg/L (ATCC25923), 2 mg/L (HPV107) and 0.5 mg/L (HPV107$_{cured}$) and of the EI verapamil at 200 mg/L (subinhibitory concentration). Controls containing solely the bacterial cultures or EtBt showed none or residual fluorescence (data not shown).

Moreover, strain HPV107$_{cured}$, without the *qacA* gene, showed a behaviour similar to the fully antibiotic-susceptible strain *S. aureus* ATCC25923, with residual efflux activity and concomitantly a higher accumulation of ethidium bromide (Figure

3.2). The residual efflux activity detected in $HPV107_{cured}$ could be the result of the basal activity of chromosomally encoded efflux pumps. These results confirmed that the activity of the efflux pump QacA was the main contributor to reduced susceptibility of the HPV107 to biocide and dyes.

Biocides are heavily used in healthcare settings for the management of nosocomial infections as well as in domestic and food industry settings [5]. Consequently, nosocomial bacteria, and increasingly community bacteria, are constantly being exposed to these daily-use compounds, raising concerns about the role of biocides as a selective pressure for the appearance and maintenance of antibiotic-resistant bacteria. Inasmuch as bacteria with reduced susceptibility to biocides could be more prone to survive in such environments, they can also create the opportunity for bacteria to develop an antibiotic-resistant phenotype, or even a MDR phenotype. Reduced susceptibility is mainly conferred by efflux pumps that have the capacity to expel from the cell interior a wide range of chemically dissimilar compounds, including one or several classes of antibiotics, and may render the cell with a MDR phenotype [11].

In the particular case of *S. aureus*, prevention of nosocomial infections is mainly conducted by the use of antiseptics and disinfectants [15]. Hence, the emergence of biocide-resistant, and possibly antibiotic-resistant bacteria can be of great importance. One such example of possible cross-resistance is the existence of plasmids that can harbor efflux pump genes that confer resistance to biocides as well as resistance determinants to antibiotics and heavy metals [11]. Previous studies have shown that the plasmid encoded *qacA/B* gene is the main efflux pump gene responsible for reduced susceptibility to biocides in *S. aureus* isolates and that it is widespread in Europe [7]. Although some data do not support a link between increased biocide resistance and antibiotic resistance, other studies highlight the potential biocide/antibiotic cross-resistance in *S. aureus*, particularly among hospital-associated MRSA strains, and potentially community-associated MRSA strains [15], owing to the common linkage of *qacA/B* and *smr* genes with β-lactamase transposons as well as to resistance determinants to gentamicin and trimethoprim, among others [14]. This was not the case for MRSA strain HPV107, since both HPV107 and $HPV107_{cured}$ showed identical levels

of resistance to gentamicin, kanamycin and trimethoprim (Table 3.1), thus excluding the presence of such resistance determinants on the plasmid carrying the *qacA* determinant.

3.1.4 Conclusions

Conjugation of a highly successful genetic background, such as that of strain HPV107, a representative of the multiresistant international MRSA Iberian clone, with a plasmid carrying the structural gene for an efflux pump that confers resistance to antiseptic and disinfectant compounds, usually employed in healthcare settings, suggests that *S. aureus* strains carrying this determinant can be more prone to survive in such environments, thereby increasing their capacity to disseminate widely in a hospital setting.

3.1.5 References

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3.2. Role of pSM39, a multiresistance plasmid harboring the *qacA* gene for biocide resistance, on the resistance phenotype of a MRSA strain

3.2.1 Introduction

The application of biocides is one of the central mainstays in practices of prevention and management of infection in healthcare settings [19]. Their widespread use has raised some concerns in the scientific community regarding their misuse and the consequent emergence of biocide-resistant bacteria [27, 31]. Other concern reflects the potential selective pressure exerted by biocides in the development of antibiotic-resistant bacteria, mainly due to the activation of promiscuous resistance mechanisms, such as multidrug efflux pumps, which are able to extrude compounds that are structurally and chemically distinct, including antibiotics, biocides, dyes and solvents [37]. In addition, some of these multidrug efflux pumps are located on mobile genetic elements such as plasmids that many times carry additional resistance determinants, revealing not only a potential cross-resistance but also co-resistance between these classes of antimicrobial compounds [37].

One of the most studied efflux systems in *S. aureus* is the QacA pump with more than 30 different classes of compounds identified as substrates, including QACs, diamidines and biguanidines [8]. The *qacA* gene is usually located on large multiresistance conjugative and non-conjugative plasmids and occurs in several staphylococci [8].

In this work we describe a plasmid isolated from a human methicillin-resistant *S. aureus* clinical isolate carrying the *qacA* gene and assess the relevance of the QacA efflux pump and of the *qacA*-carrying plasmid on the resistance profile of the MRSA clinical strain.

3.2.2 Material and Methods

Bacterial strains and growth conditions. Strain SM39 was collected from a biosopsy sample at a Portuguese hospital [14]. This strain belonged to a collection of 53 *S. aureus* clinical isolates. It presents a multidrug resistance (MDR) phenotype, with resistance to β-lactams, macrolides and aminoglycosides. *S. aureus* strain RN4220 was used as a fully-susceptible reference strain [21, 33]. Strains were grown in tryptone soya broth (TSB) or agar (TSA) (Oxoid Ltd., Basingstoke, U.K.) at 37ºC. For MIC determination, strains were grown in Mueller-Hinton broth (MHB, Oxoid) at 37ºC.

Antibiotics, biocides, dyes and efflux inhibitors. Antibiotics, biocides and dyes were purchased in powder from Sigma-Aldrich (St. Louis, MO, USA), namely: erythromycin; oxacillin; gentamicin; EtBr; acriflavine; berberine; benzalkonium chloride; tetraphenylphosphonium bromide; pentamidine isothionate salt; dequalinium chloride; cetylpyridinium chloride and cetrimide. Ciprofloxacin; chlorhexidine diacetate and hexadecyltrymethylammonium bromide (CTAB) were purchased from Fluka Chemie GmbH (Buchs, Switzerland). Norfloxacin was acquired from ICN Biomedicals Inc. (Ohio, USA). Thioridazine (TZ), chlorpromazine (CPZ) and verapamil (VER) were purchased from Sigma. All solutions were prepared in desionized water on the day of the experiment and kept protected from light.

Drug susceptibility testing. Antibiotics, biocides and dyes. MICs for antibiotics were determined by the two-fold broth microdilution method and the results evaluated according to the CLSI breakpoints [9]. MICs for biocides and dyes were also determined using the two-fold broth microdilution method. After an 18 h incubation period at 37ºC, the MIC values were recorded, corresponding to the lowest concentration of compound that presented no visible growth. **Efflux inhibitors (EIs).** MIC determination for a given compound in the presence of EIs was carried out in parallel with the MIC determination in the absence of the EI. In medium containing varying concentrations of the compound and a bacterial inoculum corresponding to the one used for MIC determination, the EIs were added at the following final concentrations: TZ (12.5 mg/L), CPZ (25 mg/L), which correspond to half of the MIC of each inhibitor. The cultures were incubated for 18 h and growth evaluated visually.

The EI was considered to have an inhibitory effect when it caused a decrease of at least four-fold on the MIC of a given compound, relatively to the original MIC of that same compound [10]. All MIC determinations were performed in triplicate.

Real-time fluorometric detection of efflux. This methodology allows the real-time fluorometric detection of the accumulation of a given efflux pump substrate (in this case, EtBr) inside the cells and its efflux, using a Rotor-Gene 3000^{TM} thermocycler, together with real-time analysis software (Corbett Research, Sidney, Australia) [46]. For EtBr efflux evaluation, cultures were grown in TSB medium at 37ºC with shaking until they reached an OD_{600nm} of 0.6. To prepare the cellular suspension, cells were collected by centrifugation at 13,000 rpm for 3 min and the pellet washed twice with a 1X PBS solution. The OD_{600nm} of the cellular suspension was then adjusted to 0.3 with 1X PBS. To prepare EtBr loaded cells, this cellular suspension was incubated with 2 mg/L EtBr (SM39) or 0.25 mg/L (RN4220 and $SM39_{cured}$) plus 200 mg/L of the efflux inhibitor VER for 60 min at 25ºC. After EtBr accumulation, the cells were collected by centrifugation and resuspended in 1X PBS to an OD_{600nm} of 0.6. Several parallel assays were then performed, corresponding to the EtBr loaded cells incubated with: (1) PBS 1X only; (2) 0.4% glucose; (3) 200 mg/L VER only; (4) 0.4% glucose plus 200 mg/L VER. The final cellular concentration in each assay corresponded to an OD_{600nm} of 0.3. The assays were then conducted in the Rotor–Gene 3000^{TM} at 37ºC, and the fluorescence of EtBr was measured (530/585 nm) at the end of every cycle of 10 sec, for a total period of 10 min. The raw data obtained was then normalized against data from non-effluxing cells (cells from the control tube with only 200 mg/L VER), at each point, considering that these correspond to the maximum fluorescence values that can be obtained during the assay. The relative fluorescence thus corresponds to the ratio of fluorescence that remains per unit of time, relatively to the EtBr loaded cells.

Multilocus sequence typing (MLST). Internal fragments of the seven genes *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqiL* were amplified by PCR using the primers and conditions described previously [11, 13]. The PCR products were then sequenced in both strands using the same set of primers and the sequence was submitted to the MLST database (www.mlst.net) in order to obtain an allelic profile and sequence type (ST) for SM39.

pSM39 sequencing and analysis. pSM39 was isolated using the the QIAGEN Plasmid Midi Kit (QIAGEN), with incubation with lysostaphin (50 mg/L) (Sigma) at 37ºC for 90 min prior to extraction. The entire sequence of pSM39 was determined by next generation sequencing at STAB-Vida, Ltd.. The assembly of the raw sequences into contigs was conducted also at STAB-Vida. The analysis of the several contigs was done using the BLASTn freeware program available at NCBI (http://blast.ncbi.nlm.nih.gov/). The following primers were used for sequencing and closing the gaps between contigs, pSM39_1, 5'-CCATTGCCATACCTCCTA-3' and pSM39_2, 5'-GCAGCTGCGGTATATGTAG-3'.

3.2.3 Results

3.2.3.1 Description of strain SM39

The plasmid pSM39 was isolated from the MRSA clinical isolate SM39, collected from a biopsy at a Portuguese hospital in 2007. This strain was the only, amongst a collection of 53 *S. aureus* isolates, carrying the efflux pump gene *qacA*. Molecular typing of the entire collection by pulsed-field gel electrophoresis (PFGE) revealed that this strain had a unique PFGE pattern. MLST typing of SM39 showed that it belongs to ST88. This clonal lineage is associated with community-onset infections, and is prevalent in Africa and Asia [7, 25]. In Europe, it has been found sporadically in several countries being correlated in somes cases with importation of CA-MRSA strains from other regions [4, 5, 12, 15, 22, 43].

3.2.3.2 Contribution of pSM39 to the resistance profile of strain SM39

Strain SM39 presented a MDR phenotype, with resistance to β-lactams, macrolides and aminoglycosides. It also presented reduced susceptibility to a large variety of QACs, like cetrimide, benzalkonium chloride, cetylpyridinium chloride, tetraphenylphosphonium bromide, dequalinium chloride, pentamidine and to the dye

EtBr (Table 3.2). These MIC values were two- to 128-fold higher than the ones presented by the susceptible reference strain *S. aureus* RN4220. To verify the contribution of the *qacA* gene and the plasmid pSM39 to this resistance, strain SM39 was cured of plasmid pSM39, yielding strain $SM39_{cured}$. The susceptibility profile of $SM39_{cured}$ towards biocides was found to be similar to RN4220 (Table 3.2), suggesting that the loss of the plasmid and, consequently of *qacA*, rendered the strain susceptible to these compounds. Regarding the antibiotics tested, no difference in susceptibility was observed between SM39 and $SM39_{cured}$, although $SM39_{cured}$ showed a reduction of the MICs of the β-lactams oxacillin and ampicillin, with no change in suscepbility profile (Table 3.2).

Table 3.2. MIC values (mg/L) of biocides, dyes and antibiotics for the strains in study in the absence and presence of efflux inhibitors (EIs).

	MIC (mg/L)								
	RN4220			SM39			$SM39_{cured}$		
	No EI	+ TZ	+ CPZ	No EI	+ TZ	+ CPZ	No EI	+ TZ	+ CPZ
Biocides and dyes									
EtBr	2	0.125	1	256	0.5	1	4	0.25	0.5
BER	64	16	8	>256	16	32	64	32	32
ACR	8	1	1	256	4	16	16	4	4
CTAB	2	0.06	1	8	<0.06	0.125	1	0.125	0.125
CET	2	0.06	1	16	<0.06	0.125	4	0.25	0.25
BAC	1	0.06	0.25	8	0.03	0.25	1	0.06	0.06
CPC	0.5	0.01	0.125	4	0.015	0.06	0.5	0.03	0.06
TPP	8	0.25	0.25	512	2	2	16	1	2
DQ	1	0.01	0.5	8	0.5	<0.25	1	0.5	0.5
CHX	0.5	0.008	0.03	1	0.06	0.125	0.5	0.03	0.06
PT	8	2	2	256	4	16	16	8	8

EtBr: ethidium bromide; BER: berberine; ACR: acriflavine; CTAB: hexadecyltrimethylammonium bromide; CET: cetrimide; BAC: benzalkonium chloride; CPC: cetylpyridinium chloride; TPP: tetraphenylphosphonium bromide; DQ: dequalinium chloride; CHX: chlorhexidine diacetate; Pt: pentamidine isothionate salt; TZ: thioridazine; CPZ: chlorpromazine. The following EI concentrations were used: 6.25 mg/L of TZ; 12.5 mg/L of CPZ. Shadowed values correspond to a decrease of ≥ 4-fold in the MICs in the presence of efflux inhibitors in comparison to the original MIC values.

Table 3.2. ***(Cont.)*** **MIC values (mg/L) of biocides, dyes and antibiotics for the strains in study in the absence and presence of efflux inhibitors (EIs).**

	MIC (mg/L)								
	RN4220			SM39			$SM39_{cured}$		
	No EI	+ TZ	+ CPZ	No EI	+ TZ	+ CPZ	No EI	+ TZ	+ CPZ
Antibiotics									
CIP	0.25	0.125	0.125	0.5	0.06	0.06	0.5	0.125	0.125
NOR	1	0.25	0.25	1	0.125	0.125	1	0.5	0.25
OXA	0.125	0.01	0.03	32	8	8	8	0.06	0.125
AMP	0.125	0.03	0.03	128	4	16	8	0.125	0.125
GEN	0.25	nd	nd	0.5	nd	nd	0.5	nd	nd
ERY	0.25	nd	nd	>256	nd	nd	>256	nd	nd

CIP: ciprofloxacin; NOR: norfloxacin; OXA: oxacillin; GEN: gentamicin; ERY: erythromycin; TZ: thioridazine; CPZ: chlorpromazine; nd: not determined. The following EI concentrations were used: 6.25 mg/L of TZ; 12.5 mg/L of CPZ. Shadowed values correspond to a decrease of ≥ 4-fold in the MICs in the presence of efflux inhibitors in comparison to the original MIC values.

3.2.3.3 Correlation of efflux to the resistance profile of SM39

To assess the efflux activity associated with *qacA* and pSM39, MIC values of the previously tested biocides were re-determined in the presence of efflux inhibitors (Table 3.2) and EtBr efflux activity followed by real-time fluorometry (Figure 3.3). Analysis of the MIC values of biocides in the presence of the inhibitors showed that the three strains suffered MIC reductions, indicating efflux activity. Reduction of MICs of β-lactams was also detected in the presence of efflux inhibitors. The overall inhibitory effect was more pronounced on the *qacA*-carrying strain, SM39, with MIC reductions of up to 512-fold, to values similar or lower than the ones for RN4220 and $SM39_{cured}$, suggesting that SM39 has a higher efflux capacity than the remaining strains. EtBr efflux assays by real-time fluorometry confirmed this hypothesis (Figure 3.3). In fact, while the control strain RN4220 presented a basal efflux activity, with a 50% loss of EtBr fluorescence over a 10 min interval, the *qacA*-carrying SM39 showed a prompt efflux activity, with a loss of 60% of EtBr fluorescence in the first minute of the assay. On the other hand, the absence of pSM39 and *qacA* resulted in a decrease of efflux

activity in $SM39_{cured}$, with the strain presenting an efflux profile similar to the one of RN4220.

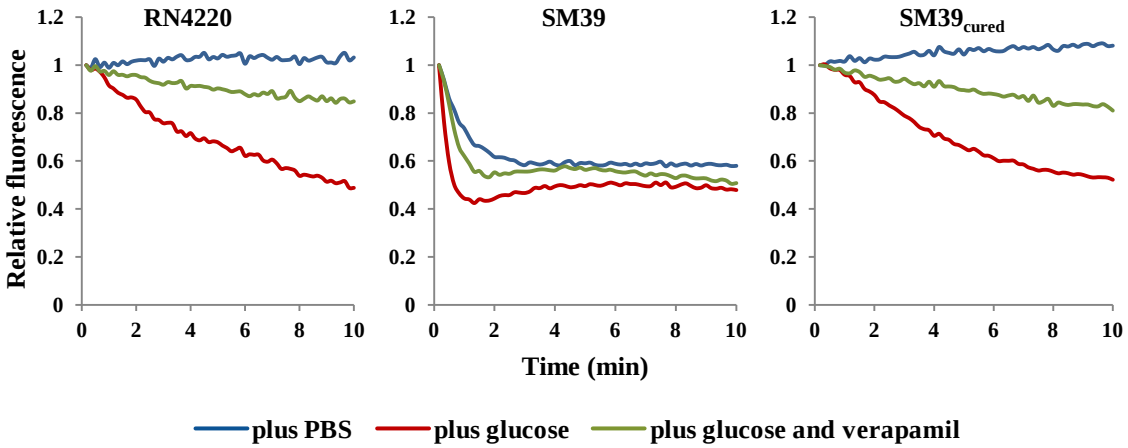

Figure 3.3. Assessment of EtBr efflux activity by real-time fluorometry of the strains RN4220, SM39 and $SM39_{cured}$. Assays were conducted in the presence of glucose 0.4% and of glucose 0.4% plus verapamil 200 mg/L. The data presented was normalized against the data obtained in conditions of no efflux (absence of glucose and presence of 200 mg/L of verapamil).

3.2.3.4 Main characteristics of pSM39

The entire sequence of pSM39 was determined by next-generation sequencing. pSM39 is a 26,036 bp plasmid, with an average G + C content of 33%. The presence of ORFs with more than 50 amino acids was verified with the ORF Finder software (http://www.ncbi.nlm.nih.gov/projects/gorf/) and 30 putative ORFs were identified (Table 3.3). Among these, BlastP analysis identified 18 genes associated to plasmid replication, maintenance functions or resistance to antimicrobials. The remaining 12 ORFs showed 88 to 100% identity with hypothetical proteins. No genes for mobilization or conjugation were detected in pSM39. The physical and genetic map of pSM39 is shown in Figure 3.4.

Table 3.3. Description of the ORFs present in pSM39 and the best match obtained with BlastP analysis.

ORF (Name)	Frame	Position (bp)	Protein size (aa)	Best match	Identity (%)
ORF1	+ 2	1097-1252	51	Hypothetical protein	90
ORF2	- 3	1632-1444	62	Truncated replication protein	100
repA	- 3	2706-1762	314	Replication protein A	100
par	+ 3	3150-3920	261	Replication-associated protein	100
ORF5	- 1	4796-4236	186	Hypothetical protein	100
qacA	- 1	6545-5001	514	MFS multidrug efflux protein QacA	100
qacR	+ 3	6723-7289	188	Transcriptional regulator QacR	100
ORF8	- 2	7909-7454	151	Hypothetical membrane protein from *Staphylococcus epidermidis*	100
Sin	- 3	8646-8035	202	Recombinase Sin from *S. epidermidis*	100
p271	- 2	9595-8780	271	ABC transporter	100
p480	- 1	11030-9588	480	Tn552 transposase	100
binL	- 3	11493-11002	163	DNA invertase BinL	100
ORF13	+ 2	11387-11584	65	Hypothetical protein	91
blaI	- 1	12239-11589	126	β-lactamase repressor protein BlaI	100
blaR1	- 3	13986-12229	585	β-lactamase regulatory protein BlaR1	100
blaZ	+ 2	14938-14444	164	Truncated β-lactamase BlaZ	100
cadC	+ 3	15501-15869	122	Cadmium efflux accessory protein	100
cadA	+ 1	15862-18045	727	Cadmium ABC efflux protein CadA	100
ORF19	- 1	17670-17897	75	Hypothetical protein	88
IS*257*	- 3	18280-18954	224	IS*257* transposase	100
ORF21	+ 2	19211-19753	180	Regulatory protein in mercury resistance operon	100
merR	+ 1	20053-20460	135	MerR regulatory protein	100
ORF23	+ 2	20477-20962	161	Hypothetical protein	100
ORF24	+ 1	20959-21639	226	Hypothetical protein	100
ORF25	- 1	21714-21977	87	Hypothetical protein	100
merT	+ 1	21727-22113	128	Mercury transporter protein MerT	100

Table 3.3. (*Cont).* Description of the ORFs present in pSM39 and the best match obtained with BlastP analysis.

ORF (Name)	Frame	Position (bp)	Protein size (aa)	Best match	Identity (%)
merA	+ 1	22171-23814	547	Mercury reductase protein MerA	100
merB	+ 1	23896-24546	216	Alkylmercury lyase protein MerB	100
ORF29	- 1	24741-25289	182	Hypothetical protein	100
IS*257*	- 3	25375-13	224	IS*257* transposase	100

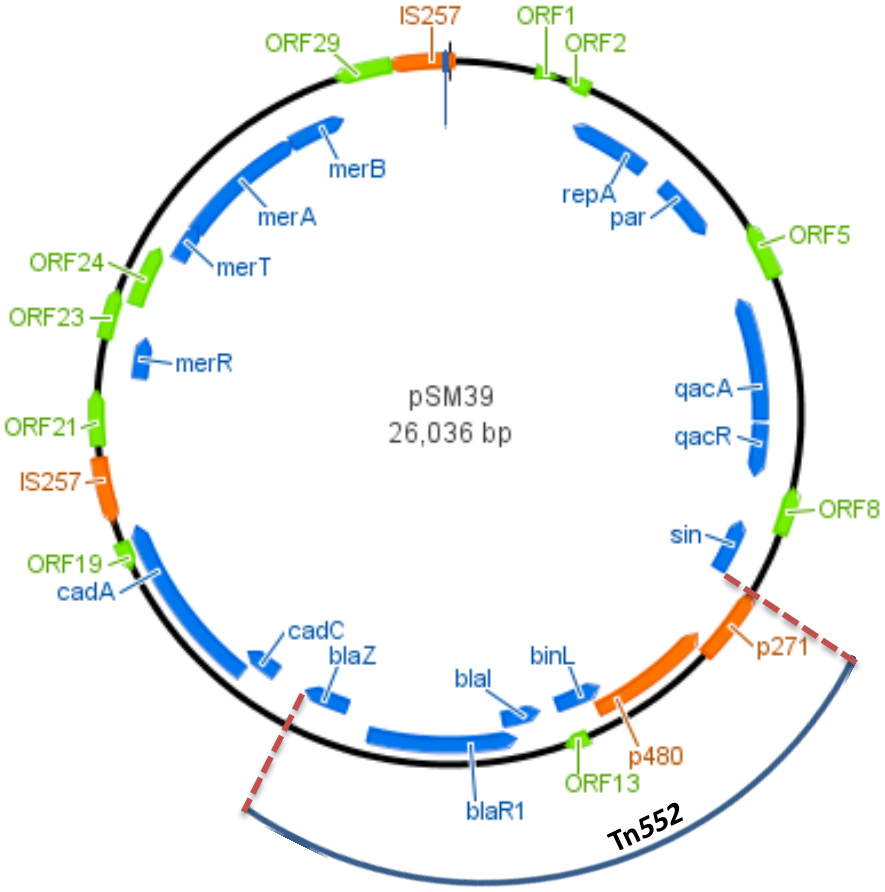

Figure 3.4. Physical map and genetic organization of the *S. aureus* plasmid pSM39. Identified genes are indicated by blue arrows; ORFs of unknown function are indicated by green arrows; transposase genes are indicated by orange arrows.

ORF3 was found to encode a RepA_N replication initiation protein. Recently, plasmid classification systems have been established that use the sequence of the *rep* gene to form *rep* gene families [20] and plasmid groups (each with a unique combination of *rep* genes) [30]. According to these classification systems, the *rep* gene

from pSM39 is part of the rep_{22} gene family and pSM39 belongs to the *S. aureus* plasmid group pGSA11. ORF4 was initially identified as a replication-associated protein and further analysis revealed that it has 65% amino acid identity to the *par* gene of plasmid pSK1. This gene has been shown to participate in plasmid maintenance by enhancing plasmid segregational stability [41]. Other genes with plasmid maintenance functions were also encountered, namely the recombinases Sin (ORF9) and Bin (ORF12).

Plasmid pSM39 is mainly composed by determinants of resistance to antimicrobials. Of the 30 ORFs identified, 11 were associated with resistance to antibiotics, biocides and heavy-metals. As expected, pSM39 carried the *qacA* gene (ORF6) and its regulator *qacR* (ORF7). As already detected in several staphylococcal plasmids, the *qacA* and *qacR* genes were located next to a full copy of the transposon Tn*552* that comprises two transposases genes, *p271* (ORF11) and *p480* (ORF10), the recombinase *bin* (ORF12) and the β-lactam resistance genes *blaI* (ORF14), *blaR1* (ORF15) and *blaZ* (ORF16) [3, 40]. Among the determinants for heavy-metal resistance, ORF18 and ORF17 were identified as the cadmium efflux pump CadA from the ABC superfamily and its accessory protein CadC, respectively. We could also identify the *mer* operon, harboring genes for mercuric resistance that are flanked by two copies of the IS*257*.

Analysis of the entire sequence of pSM39 revealed a high homology with plasmids from the β-lactamase/heavy-metal resistance family, such as plasmid pI258 [23], pSK57 and pSK23 [39]. All these plasmids harbor the *mer* operon and the *cadCA* genes for mercuric and cadmium resistance, respectively. The region where these genes are located is nearly identical in all three plasmids and pSM39. However, these plasmids differ in the Tn*552* region. While pSM39 harbors a full copy of Tn*552*, pI258 and pSK57 carry the truncated form ΔTn*552* that lacks the transposases genes and pSK23 carries the truncated variant Tn*552*Δ lacking the *bla* genes [39]. Another region of divergence is located between the *qac* genes and Tn*552* (Figure 3.5). The *qacA* and *qacR* genes are located upstream of the Tn*552*, with the *sin* gene and ORF8 in between. Surprisingly, blast analysis for ORF8 revealed that it encodes a putative membrane protein with 100% identity to a protein from plasmid pSK105 of *S. epidermidis*

(accession no. YP_006939293). No homology was found between ORF8 and any protein from *S. aureus*. Also, the *sin* gene of pSM39 shares 100% identity with the *sin* gene of pSK105 and only 94% identity with other *sin* genes from *S. aureus*. Moreover, the region of identity between the two plasmids extends by 4.7 kb, from the *sin* gene up to the *par* gene, comprising the *qac* genes. These findings suggest that this region of pSM39 may have been acquired from a *S. epidermidis* plasmid, such as pSK105.

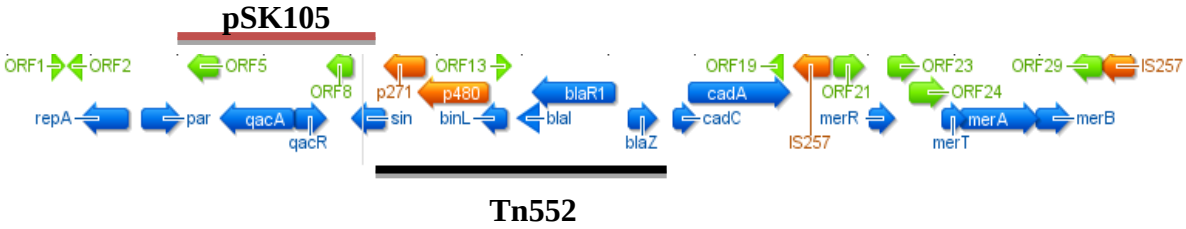

Figure 3.5. Genetic organization of pSM39 and the location of the transposon Tn*552* (black line) and of the region with high homology to the *S. epidermidis* plasmid pSK105 (red line).

3.2.4 Discussion

Reduced susceptibility to antiseptics and disinfectants has been associated with carriage of the *qacA* gene. It has been shown that its product, the efflux pump QacA can extrude from the *S. aureus* cell a wide variety of chemical compounds that are dissimilar in their structure [8]. In the last decade, new interest has been shown in antiseptic resistance determinants, such as *qacA*, as the scientific community becomes more aware of the potential impact of biocides on emergence of strains with reduced susceptibility to these agents but also to antibiotics [27, 31]. Efforts have been made to assess the carriage rate of antiseptic resistance genes in clinical isolates of *S. aureus* around the world. The reported prevalence rates found for *qacA* and the closely-related *qacB* gene differ geographically, with prevalences ranging from 0 to 73% in several Asian countries [2, 35], from 6% to 42% in European countries [16, 29, 42, 45], 13% in Tunisia [48] and 2% in Canada [26]. No data has been published so far for Portugal, and though the main focus of this study was not the determination of the prevalence rate of *qacA*, a single isolate out of 53 *S. aureus*, isolated during a four month period in one of the largest Portuguese hospitals, harbored the *qacA* gene, that is, with an occurrence rate

of 1.9% amongst the collection tested, which is lower than the values reported to date. However, larger screening studies should be performed to fully ascertain the prevalence rate of the *qacA/B* genes in Portugal, not only in isolates from human origin but also from animal original and representative of both *S. aureus* and coagulase-negative staphylococci.

Notwithstanding, the single isolate harbouring *qacA*, SM39, was distinct from the remaining *S. aureus* collection, showing a different PFGE profile. Molecular typing revealed it to belong to the clonal lineage ST88 associated with infections with onset in the community [5, 43]. This clonal lineage is reported to occur only sporadically in Europe and has been associated, in some cases, with importation from other countries [4, 12]. To our knowledge, this is the second isolate from this lineage to be reported in Portugal [1]. Surprisingly, in a multicenter study carried out to analyze the population structure of community-associated MRSA (CA-MRSA) strains in Europe did not report the finding of a ST88 lineage [38].

The work described in this Chapter to survey the actual role of *qacA* and plasmid pSM39 on the resistance phenotype presented by strain SM39 correlated carriage of these elements with a reduced susceptibility profile to biocides. In fact, SM39 displayed higher MIC values to all the compounds tested, including the QACs benzalkonium chloride, cetrimide and cetylpyrydinium chloride, the diamidine pentamidine and to the dye EtBr when compared to the reference strain RN4220 or the plasmid cured derivative, $SM39_{cured}$. Despite the lack of guidelines to establish cut-off values to evaluate the susceptibility of bacteria to biocide agents, the resistance levels presented by SM39 are, in general, higher than the ones found in literature for strains carrying other antiseptic resistance genes, like the efflux pump gene *smr*, which is discussed in Chapter 4 of this Thesis. Thus, when comparing these efflux systems, one may say that QacA conveys high-level resistance to biocides. Tentative biocide epidemiological cut-off (ECOFF) values for benzalkonium chloride and chlorhexidine, based on MICs distributions, have been proposed for *S. aureus*, namely 4 mg/L and 2 mg/L, respectively [6]. These values indicate that strains displaying higher values may present a resistance mechanism towards these antimicrobials. SM39 presents MICs of 8 mg/L for benzalkonium chloride and 1 mg/L for chlorhexidine, indicating that the

chlorhexidine cut-off value may not be appropriate for SM39. Nevertheless, further harmonization of methodology to assess biocide susceptibility is still required and may affect these parameters.

Determination of MICs in the presence of efflux inhibitors together with fluorometric assays for the strains in study demonstrated that the resistance profile of SM39 towards biocides is mediated by QacA efflux activity. Inasmuch, as the presence of efflux inhibitors could reduce the values of MICs for SM39 to the levels presented by the susceptible strain RN4220, the fluorometric assays also demonstrated that SM39 possesses a prompt efflux activity which is lost with the curing of the plasmid pSM39. These findings are in accordance with the recent data reported by Furi *et al*. that found a change in the MIC mode of benzalkonium chloride and chlorhexidine in human clinical isolates correlated with the presence of *qac* genes, although not a change in minimum bactericidal concentrations [16].

The determination of MIC values of β-lactams for the SM39 and $SM39_{cured}$ strains suggests that a resistance determinant for this class of antibiotics is present in the plasmid. Sequencing the plasmid revealed that pSM39 carried the *blaZ* gene, encoding a β-lactamase. The presence of this determinant can be correlated to the decrease of the ampicillin MIC in $SM39_{cured}$, but not to oxacillin, as this antibiotic is not susceptible to β-lactamases. This finding could be explained by hyperproduction of β-lactamase that could confer some level of resistance to oxacillin [28, 32], or by a co-regulation exerted by the *blaZ* regulators, *blaI* and *blaR1,* on the expression of the *mecA* gene, and consequent production of PBP2a [17, 18, 24]. However, these hypotheses fail to explain the effect of efflux inhibitors on the MICs of ampicillin and oxacillin. This observation may be the result of a synergistic effect between phenothiazines (thioridazine and chlorpromazine) and β-lactams, as a recent study showed that thioridazine has the ability to sensitize MRSA strains to antimicrobials that target the synthesis of peptidoglican by modulation of genes involved in the cell wall biosynthesis [44].

We determined the entire sequence of pSM39. This is a large non-conjugative multiresistance plasmid from the family of the β-lactamase/heavy-metal resistance plasmids that also includes pI258 and pSK23, the latter one harboring the *qacB* gene [20]. A recent study by Shearer *et al.* observed a prevalence of non-conjugative

multiresistance plasmids with > 20 kb in staphylococci, indicating that transduction may be relevant for the transfer of these plasmids [39]. Such is the case for the non-conjugative plasmid pTZ2162 that harbors the *qacB* gene [34]. Further studies are required to establish if the transfer by transduction of pSM39 can also occur. According to the more recently established classification systems, belongs pSM39 belongs to the *S. aureus* plasmid group pGSA11 [20, 30]. This group comprises plasmids that are mainly involved in resistance to antimicrobials, carrying determinants for resistance to antibiotics (β-lactams, aminoglycosides, macrolides, trimethoprim), heavy-metals (cadmium, mercury, arsenate), and biocides. These large non-conjugative multiresistance plasmids are very prevalent among staphylococci, probably as the result of the selective pressure imposed by these antimicrobials not only in healthcare settings but also in animal-associated settings and in the environment [30, 39]. They also highlight the potential co-selection between the several antimicrobial compounds on the maintenance of these plasmids as they provide to the cell advantages to different noxious environments. One example of potential co-selection and co-resistance, already described for several plasmids of several staphylococcal species and isolated from humans and animals, is the common co-occurrence of the *qacA/B* genes with the transposon Tn*552*, and thus resistance to β-lactams [3, 40]. This co-occurrence was also observed in pSM39, and more importantly, evidence was found that this plasmid may result from a rearrangement between a *S. aureus* plasmid and a *S. epidermidis* pSK105-like plasmid, emphasizing the role of other staphylococci as a reservoir for the transfer of resistance genes.

3.2.5 Conclusions

We report here the occurrence of a new large non-conjugative multiresistance plasmid, pSM39, in a MRSA strain, SM39, harboring the efflux pump gene *qacA*. We provide evidence that *qacA* and pSM39 were essential for the SM39 phenotype of reduced susceptibility towards quaternary ammonium compounds, diamidines and chlorhexidine; compounds widely used as antiseptic and disinfectants in hand formulations and patient decolonization in healthcare settings. These findings are

supported by some studies that have reported increased *qacA* carriage rates in clinical isolates over time or following introduction of decolonization protocols [36, 47]. Furthermore, selection of *qacA* could also potentiate the selection of resistance to antibiotics and other antimicrobial agents due to their co-localization on the same plasmid. Also, evidence of the origin of pSM39 in the rearrangement between plasmids of *S. aureus* and *S. epidermidis* origin brings to light the role of other staphylococci as reservoirs for additional resistance determinants.

3.2.6 References

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CHAPTER 4

The role of Smr in efflux-mediated resistance

4. The role of Smr in efflux-mediated resistance

This Chapter contains data published in:

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Summary

Biocides are of major importance in the prevention and control of infections, particularly in healthcare settings. Increased resistance to biocides bestowed by efflux pumps is an important mechanism for bacteria to persist in hostile environments. Screening of a collection of 52 *S. aureus* clinical isolates for the presence of several efflux pump genes (Chapter 5) allowed the identification of a single MRSA isolate, SM52, carrying the determinant *smr* within plasmid pSM52. In this Chapter, we present the data on the characterization of pSM52, a 2.8 kb plasmid containing seven putative ORFs, including the replication initiation gene *rep52* and a type 2 *smr* cassette-like structure, comprising *smr* and a partial duplicate, Δ*smr*. According to recently published classification systems, pSM52 belongs to the rep_{13} family and group pGSA5 of *S. aureus* plasmids. Sequence analysis suggests that pSM52 may have resulted from an arrangement between different antibiotic/antiseptic-resistance staphylococcal plasmids. Smr efflux activity was correlated with increased resistance to quaternary ammonium compounds, such as cetrimide, benzalkonium chloride and cetylpyridinium chloride, as well to the dye EtBr. The occurrence of this newly described plasmid in a human MRSA isolate harboring an antiseptic resistance gene, *smr*, strengthens the importance of plasmid-encoded efflux systems in the maintenance and dissemination of strains in the hospital environment as well as in the community.

4.1. Introduction

The use of compounds with antiseptic and disinfectant properties, known as biocides, has a central role in the prevention and control of infections in healthcare settings [15, 24]. In the last decades, the application of these biocides has spread out to the community, becoming of general use either in domestic households as well as in industrial facilities [10]. Although the employment of these compounds is essential for infection control, their use has raised concerns regarding the selection of biocide-resistant strains, and most importantly, the cross-selection of antibiotic-resistant strains [21]. Underlying these concerns is, among other factors, the existence of bacterial

resistance mechanisms, such as multidrug efflux pumps, with the ability to extrude from the cell, different types of antimicrobial compounds (including biocides and/or antibiotics) and thereby conferring multidrug resistance phenotypes.

In the particular case of *Staphylococcus aureus*, an important nosocomial pathogen, efflux-mediated resistance towards biocides has been mainly associated with plasmid-encoded efflux pumps, such as QacA/B and Smr, although chromosomally-encoded efflux pumps may also be involved in biocide resistance [27]. The efflux pump Smr (also known as QacC) uses the proton motive force to carry out the extrusion of quaternary ammonium compounds (e.g., the disinfectant benzalkonium chloride), the dye ethidium bromide and phosphonium derivatives (e.g. tetraphenylphosphonium bromide) [11]. This pump is a small protein of 107 amino acids with four transmembrane segments that belongs to the small multidrug resistance (SMR) protein family [3]. Its coding gene, *smr*, is found either in small nontransmissable plasmids (e.g. pSK89) or in large conjugative plasmids (e.g. pSK41). The *smr* gene is located in a cassette-like structure that presents some diversity, with three different arrangements (types 1 to 3) described to date [1, 4]. In the present study we characterize a plasmid, isolated from a human methicillin-resistant *S. aureus* (MRSA) clinical isolate, which carries the *smr* determinant, and evaluate the role played by the Smr pump on the resistance of this clinical MRSA strain towards several antimicrobial compounds.

4.2. Material and Methods

Bacterial strains and growth conditions. Strain SM52 was isolated from pus in a Portuguese hospital (Lisbon, Portugal) and the unique harboring the *smr* gene amongst a collection of 52 ciprofloxacin-resistant *S. aureus* samples isolated in this hospital over a four-month period [7]. It is a MRSA strain with a multidrug resistance phenotype, showing resistance to β-lactam antibiotics, fluoroquinolones, aminoglycosides and chloramphenicol. *S. aureus* strain RN4220 was used as a drug-susceptible control and as a recipient for the electrotransformation experiments [19, 25]. For culture growth, strains were grown in tryptone soya broth (TSB) or agar (TSA) (Oxoid Ltd., Basingstoke, UK) at 37ºC. For MIC determination, strains were grown in Mueller-Hinton broth (MHB, Oxoid) at 37ºC.

Antibiotics, biocides, dyes and efflux inhibitors. Antibiotics, biocides and dyes were purchased in powder from Sigma-Aldrich (St. Louis, MO, USA), namely: erythromycin; oxacillin; tetracycline; gentamicin; ethidium bromide (EtBr); acriflavine; berberine; benzalkonium chloride; tetraphenylphosphonium bromide; pentamidine isothionate salt; dequalinium chloride; cetylpyridinium chloride and cetrimide. Ciprofloxacin; chlorhexidine diacetate and hexadecyltrymethylammonium bromide (CTAB) were purchased from Fluka Chemie GmbH (Buchs, Switzerland). Norfloxacin was acquired from ICN Biomedicals Inc. (Ohio, USA). Thioridazine (TZ), chlorpromazine (CPZ) and verapamil (VER) were purchased from Sigma. All solutions were prepared in desionized water on the day of the experiment and kept protected from light.

Drug susceptibility testing. Antibiotics, biocides and dyes. MICs for antibiotics were determined by the two-fold broth microdilution method and the results evaluated according to the CLSI breakpoints [6]. MICs for biocides and dyes were also determined using the two-fold broth microdilution method. After an 18 h incubation period at 37ºC, the MIC values were recorded, corresponding to the lowest concentration of compound that presented no visible growth. **Efflux inhibitors (EIs).** MIC determination for a given compound in the presence of EIs was carried out in parallel with the MIC determination in the absence of the EI. In medium containing varying concentrations of the compound and a bacterial inoculum corresponding to the one used for MIC determination, the EIs were added at the following final concentrations: TZ (12.5 mg/L), CPZ (25 mg/L) and VER (200 mg/L), which correspond to half, or lower, the MIC of each inhibitor. The cultures were incubated for 18 h and growth evaluated visually. The EI was considered to have an inhibitory effect when it caused a decrease of at least four-fold on the MIC of a given compound, relatively to the original MIC of that same compound [7]. All MIC determinations were performed in triplicate.

Electrotransformation. Plasmid pSM52 was isolated with the QIAGEN Plasmid Midi Kit (QIAGEN), with the following modification: prior to extraction, cells were incubated with lysostaphin (50 mg/L) (Sigma) at 37ºC for 90 min, as previously described [4]. The plasmid preparation was analyzed by electrophoresis on a 0.7% agarose gel and quantified by comparison with the molecular weight marker

lambda/HindIII DNA marker (Fermentas Inc., Ontario, Canada). Electrocompetent cells of *S. aureus* RN4220 were prepared as described [18]. Briefly, an overnight culture was diluted 1:50 in TSB and grown until an optical density at 600 nm (OD_{600nm}) of 0.5-0.6. Cells were then collected by centrifugation and washed sequentially with 1X, 0.5X and 0.01X volume of sucrose 0.5M. Aliquots of 0.2 mL of electrocompetent cells were immediately used or stored at –70ºC. For electrotransformation, an aliquot of 0.05 mL of electrocompetent cells was mixed with 0.5 µg of pSM52 in a 0.2-cm Gene Pulser cuvette (Bio-Rad, Hercules, CA, USA) and the mixture incubated on ice for 15 min. Afterwards, cells were electroporated by a single 2.5 msec pulse in a Gene Pulser and Pulser Controller (Bio-Rad) set at 25 µF, 2.5 kV and 100 Ω. One mL of SMMP medium [5] was added to the cuvette and cells incubated at 37ºC for one h. For selection of electrotransformants, the cells were serially diluted in phosphate buffered saline (PBS) and plated in Penassay broth supplemented with 5 mg/L of EtBr. Colony growth was verified after 24 to 48 h of incubation at 37ºC. Additionally, the differentiation between colonies lacking the *smr* gene and colonies which carry it was done by evaluating the fluorescence emitted by colonies in a UV-transiluminator (Uvitec Limited, Cambridge, UK). Colonies formed by cells which carry the *smr* gene, will accumulate less EtBr, showing a lower emission of fluorescence than colonies lacking the *smr* gene. Thus, non-fluorescent colonies, which potentially carry the *smr* gene, were selected for further analysis by plasmid isolation and screening of the *smr* gene by PCR.

Real-time fluorometric detection of efflux. This methodology allows the real-time fluorometric detection of the accumulation of a given efflux pump substrate (in this case, EtBr) inside the cells and its efflux, using a Rotor-Gene 3000^{TM} thermocycler, together with real-time analysis software (Corbett Research, Sidney, Australia) [30]. For EtBr efflux evaluation, cultures were grown in TSB medium at 37ºC with shaking until they reached an OD_{600nm} of 0.6. To prepare the cellular suspension, cells were collected by centrifugation at 13,000 rpm for 3 min and the pellet washed twice with a 1X PBS solution. The OD_{600nm} of the cellular suspension was then adjusted to 0.3 with 1X PBS. To prepare EtBr loaded cells, this cellular suspension was incubated with 1 mg/L EtBr (SM52 and RN4220:pSM52) or 0.25 mg/L (RN4220) plus 200 mg/L of the efflux inhibitor VER for 60 min at 25ºC. After EtBr accumulation, the cells were collected by centrifugation and re-suspended in 1X PBS to an OD_{600nm} of 0.6. Several parallel assays

were then performed, corresponding to the EtBr loaded cells incubated with: (1) PBS 1X only; (2) 0.4% glucose; (3) 200 mg/L VER only; (4) 0.4% glucose plus 200 mg/L VER. The final cellular concentration in each assay corresponded to an OD_{600nm} of 0.3. The assays were then conducted in the Rotor–Gene 3000^{TM} at 37ºC, and the fluorescence of EtBr was measured (530/585 nm) at the end of every cycle of 10 sec, for a total period of 10 min. The raw data obtained was then normalized against data from non-effluxing cells (cells from the control tube with only 200 mg/L VER), at each point, considering that these correspond to the maximum fluorescence values that can be obtained during the assay. The relative fluorescence thus corresponds to the ratio of fluorescence that remains per unit of time, relatively to the EtBr loaded cells.

Multilocus sequence typing (MLST). Internal fragments of the seven genes *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqiL* were amplified by PCR using the primers and conditions described previously [8, 9]. The PCR products were then sequenced in both strands using the same set of primers and the sequence was submitted to the MLST database (www.mlst.net) in order to obtain an allelic profile and sequence type (ST) for SM52.

pSM52 sequencing and analysis. The entire sequence of pSM52 was determined by primer-walking, using as a starting point the following internal primers for the *smr* gene; smr_fw, 5'- ATAAGTACTGAAGTTATTGGAAGT and smr_rv, 5'- TTCCGAAAATGTTTAACGAAACTA [4]. The sequence obtained was analysed with the freeware program BioEdit v. 7.0.9.0. Multiple sequence alignments were carried out with the program ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and the ORF Finder Program at the National Center for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi) site was used for prediction of open reading frames (ORFs). The nucleotide and deduced amino acid sequences were analyzed using BLASTn and BLASTP freeware programs available at NCBI. The entire nucleotide sequence of plasmid pSM52 was deposited in the GenBank database under the accession number JX898993.

4.3. Results

4.3.1. Description of pSM52

Plasmid pSM52 was isolated from SM52, a MRSA strain isolated in a Portuguese hospital with a MLST profile corresponding to ST8, a lineage related to community-associated MRSA strains, such as USA300, predominant in the USA and increasingly present in different European countries, including Portugal [28]. Sequencing by primer-walking revealed that pSM52 is 2,782 bp in length, with an average G + C content of 28.65%. The physical and genetic map of pSM52 is shown in Figure 4.1. Sequence analysis with ORF Finder predicted the presence of seven putative ORFs with more than 50 amino acids (Table 4.1). BlastP analysis of these seven ORFs allowed their characterization. ORF1 and ORF2 showed high homology (92% and 89 %, respectively) with Cop proteins from *Staphylococcus sciuri*, which are involved in the regulation of plasmid copy number and were denominated *cop1* and *cop2*, respectively. ORF3, denominated *rep52*, encodes a putative replication initiation protein (Rep), designated Rep52, that presents 99.4% homology to the Rep protein found in an integrated plasmid in the chromosome of *Streptococcus pneumoniae* strain CGSP14 (GenBank accession no. ACB90543), that carries the *cat* gene for chloramphenicol resistance. It also shows high homology with the Rep proteins of the *cat*-carrying plasmids pSCS34 of *Staphylococcus sciuri* (99.3%) [12] and pC194 of *S. aureus* (98.7%) (GenBank accession nos. EU602348 and V01277, respectively) [14]. Upstream of the *rep52* gene is located the sequence *dso*, involved in the double-strand replication of rolling-circle replicating (RCR) plasmids that comprises the highly conserved nick site and the Rep binding site [26]. ORFs 4 and 5 showed high homology (89% and 92%, respectively) to *S. aureus* hypothetical proteins with no function assigned yet. ORF 6 (*smr* gene) and ORF7 (Δ*smr*, a partial duplicate of *smr*) were found to be transcribed in opposite direction from the remaining ORFs. These two genes were found in a cassette-like structure, showing 100% identity to the ones present in the *Staphylococcus epidermidis* plasmid pSK108 [20], *Staphylococcus warneri* pPI-2 [2] and *S. aureus* pKH8 [16]. This structure consists of the *smr* gene flanked by the direct repeats, $DR2_c$, $DR1_c$ and ‘$DR1_c$ (comprising 19 nucleotides of $DR1_c$) (Figure 4.1) and corresponds to a

type 2 *smr* gene cassette [1]. Immediately downstream of *smr* is located $DR2_c$ that encloses the palindromic sequence sso_A involved in the lagging-strand replication of RCR plasmids [26]. Upstream of *smr* lies $DR1_c$. Between $DR1_c$ and *smr* is located the partial duplicate Δ*smr* and the sequence '$DR1_c$, suggesting that this region was originated through an event that duplicated 142 nucleotides of the *smr* gene and its promoter region, including the first 11 nucleotides of the 5' end of $DR1_c$.

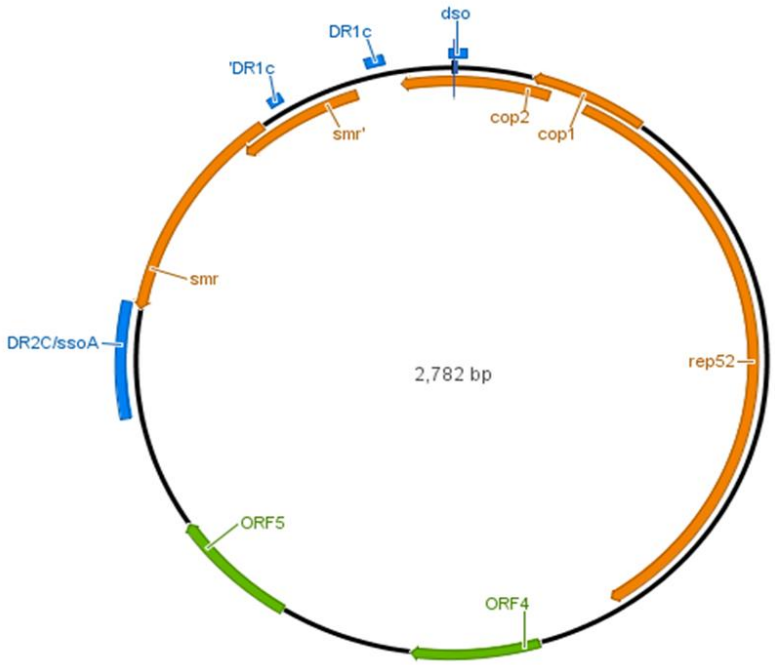

Figure 4.1. Physical map and genetic organization of the *S. aureus* plasmid pSM52. The ORFs *smr*, Δ*smr*, *cop1*, *cop2* and *rep52* are indicated by the orange arrows. The ORF4 and ORF5 are indicated by green arrows. The blue boxes correspond to the regions *dso*, $DR1_c$, '$DR1_c$ and $DR2_c$ (sso_A).

The pSM52 features suggest that this plasmid is a new member of the pC194-family of small RCR plasmids, recently re-classified as rep_{13} family, namely the high conservation of the replication initiation protein Rep52 when compared to other Rep proteins from the pC194-family and the location of the resistance gene cassette, between the nick site and the *ssoA* sequence [17, 26].

Table 4.1. Description of the ORFs present in pSM52 and the best match obtained with the BlastP analysis.

ORF (Name)	Frame	Position (bp)	Protein size (aa)	Best match
1 (*cop1*)	-1	280-122	53	92% Cop protein *S. sciuri* (regulation of plasmid copy number)
2 (*cop2*)	-3	2679-143	70	89% Cop protein *S. sciuri* (regulation of plasmid copy number)
3 (*rep52*)	+2	206-1141	311	99.4% Replication initiaton protein (*Streptococcus pneumoniae* CGSP14 chromosome - Tn*2008*)
4 (ORF4)	+2	1271-1444	57	89% Hypothetical protein from *S. aureus*
5 (ORF5)	+2	1646-1822	58	92% Hypothetical protein from *S. aureus*
6 (*smr*)	-3	2495-2172	107	100% Smr efflux pump
7 (Δ*smr*)	-2	2637-2458	59	100% to the first 32 amino acids of the Smr efflux pump

4.3.2. Antimicrobial susceptibility profiles

Strain SM52 showed a resistance phenotype to multiple classes of antibiotics, including fluoroquinolones and β-lactams (Table 4.2). MIC determination also revealed that SM52 presented increased resistance towards quaternary ammonium compounds, such as cetrimide, CTAB, cetylpyridinium chloride, benzalkonium chloride and to the dye ethidium bromide, when compared to the fully-susceptible reference strain RN4220. In order to ascertain the actual contribution of the Smr pump to the overall resistance phenotype of SM52, pSM52 was introduced into strain RN4220, yielding RN4220:pSM52. When compared to the parental strain, RN4220:pSM52 presented a four-fold increase in the MICs of ethidium bromide, berberine, CTAB, cetrimide, cetylpyridinium chloride and benzalkonium chloride (Table 4.2). Also, those MICs were similar to the ones presented by SM52 and are in accordance to MIC values described in literature for *smr*-carrying strains and strengthen the role of Smr in low-

level resistance to antiseptics and disinfectants in *S. aureus*. Regarding antibiotic susceptibility, RN4220:pSM52 remained susceptible to all the antibiotics tested, with no alteration on the MIC values compared to RN4220, thus showing that the SM52 antibiotic resistance profile was not conveyed by Smr.

Table 4.2. MIC values (mg/L) of antibiotics, biocides and dyes for the strains in study in the absence and presence of efflux inhibitors (EIs).

	MIC (mg/L)[1]								
	SM52			RN4220			RN4220:pSM52		
	No EI	+ TZ	+ CPZ	No EI	+ TZ	+ CPZ	No EI	+ TZ	+ CPZ
Biocides and dyes									
EtBr	16	1	2	2	0.125	1	8	0.25	2
BER	>256	64	128	64	16	8	>256	128	128
CTAB	8	1	1	2	0.06	1	16	0.06	4
CET	8	2	2	2	0.06	1	8	0.06	0.5
BAC	2	0.25	0.25	1	0.06	0.25	2	0.06	1
CPC	2	0.03	0.06	0.5	0.01	0.125	4	0.01	0.03
TPP	16	1	2	8	0.25	0.25	8	0.25	0.5
DQ	1	0.5	0.5	1	0.01	0.5	1	0.125	0.125
CHX	0.5	0.125	0.06	0.5	0.008	0.03	0.5	0.01	0.03
ACR	16	4	1	8	1	1	8	1	1
PT	8	4	4	8	2	2	8	2	2
Antibiotics									
CIP	16	8	8	0.25	0.125	0.125	0.25	0.125	0.125
NOR	64	32	32	1	0.25	0.25	1	0.25	0.25
OXA	8	nd	nd	0.125	nd	nd	0.125	nd	nd
GEN	1	nd	nd	0.25	nd	nd	025	nd	nd
ERY	0.5	nd	nd	0.25	nd	nd	0.5	nd	nd
TET	0.25	nd	nd	0.25	nd	nd	0.25	nd	nd

EtBr: ethidium bromide; BER: berberine; CTAB: hexadecyltrimethylammonium bromide; CET: cetrimide; BAC: benzalkonium chloride; CPC: cetylpyridinium chloride; TPP: tetraphenylphosphonium bromide; DQ: dequalinium chloride; CHX: chlorhexidine diacetate; ACR: acriflavine; Pt: pentamidine isothionate salt; CIP: ciprofloxacin; NOR: norfloxacin; OXA: oxacillin; GEN: gentamicin; ERY: erythromycin; TET: tetracycline; TZ: thioridazine; CPZ: chlorpromazine. [1] The following EI concentrations were used: 6.25 mg/L of TZ; 12.5 mg/L of CPZ. Shadowed values correspond to a decrease of ≥ 4-fold in the MICs in the presence of efflux inhibitors in comparison to the original MIC.

4.3.3. Assessment of efflux activity

The role of efflux in the increased biocide resistance phenotype of SM52 and RN4220:pSM52 was evaluated by MIC determination in the presence of efflux inhibitors and real-time fluorometric assays. MICs of the compounds previously identified as Smr substrates were reduced two- to 16-fold in the presence of the phenothiazines thioridazine (TZ) and chlorpromazine (CPZ), to values similar or lower to the ones presented by the susceptible strain RN4220 in the absence of efflux inhibitors (Table 4.2). MIC reduction in the presence of the inhibitor verapamil (VER) was less significant (data not shown). These results confirmed that efflux is the main component of the resistance to biocides in strains SM52 and RN4220:pSM52, and this finding was complemented further by the fluorometric detection of ethidium bromide efflux in these strains (Figure 4.2). In particular, it could be observed that strains SM52 and RN4220:pSM52, which carry the *smr* gene, presented a pronounced efflux activity, showing an 80% decrease in fluorescence during the first two minutes of the assay. In opposition, strain RN4220, lacking the *smr* gene, showed only a basal efflux activity.

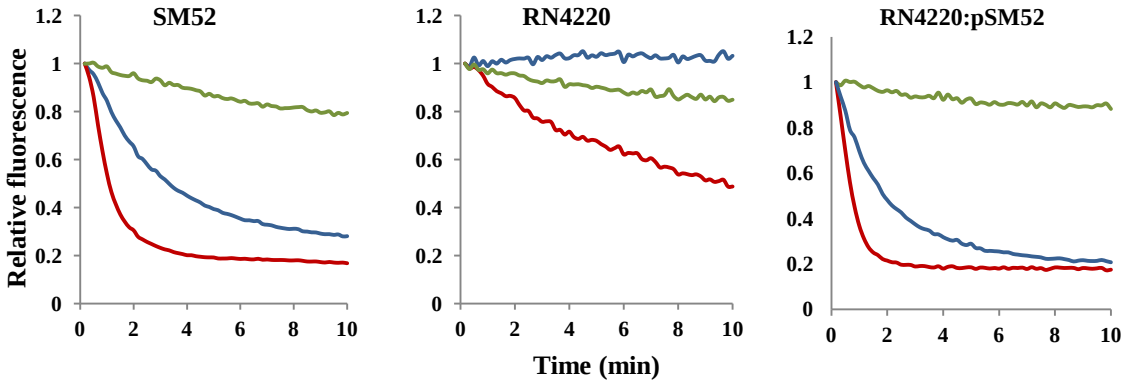

Figure 4.2. Assessment of efflux activity by fluorometry of the strains SM52, RN4220 and RN4220:pSM52. Assays were conducted in the presence of glucose 0.4% and of glucose 0.4% plus verapamil (VER) 200 mg/L. The data presented was normalized against the data obtained in conditions of no efflux (absence of glucose and presence of 200 mg/L of VER).

4.4. Discussion

Amongst the *S. aureus* plasmid-encoded efflux pumps that confer resistance to antiseptics and disinfectants, Smr has the capacity to bestow a lower level of resistance, in contrast with the QacA/B efflux system. Also, the *smr* gene is most commonly found in small staphylococcal plasmids containing a single resistance gene, such as pKH8 and pSK108, whereas *qacA/B* is commonly found on large conjugative plasmids that carry multiple antibiotic resistance determinants [13]. For this reason, the importance of Smr and consequently of these small antiseptic-resistance plasmids on the maintenance and persistence of *S. aureus* strains in healthcare settings has been overlooked. Most of the studies on *smr* and other plasmid-encoded antiseptic resistance genes are from isolates of animal origin or from industry facilities [4, 22]. The actual contribution of Smr to the resistance phenotype in these isolates is still poorly characterized, as well as the diversity of plasmids carrying it. In our earlier study, we found that strain SM52 was the only one to carry the *smr* gene among a collection of 52 ciprofloxacin-resistant *S. aureus* clinical isolates [7]. Albeit the propose of that earlier study was not to survey the prevalence of plasmid encoded efflux determinants, the low occurrence of *smr* observed is in accordance with other studies conducted with European clinical isolates, where prevalence values of 6-8% have been reported [13, 29].

In this work we describe a new plasmid carrying the *smr* gene, pSM52, isolated from a MRSA isolate presenting resistance to biocides. pSM52 belongs to the rep_{13} family (which comprises the formerly pC194-family) of small RCR plasmids, which can be found in several staphylococcal species. The plasmids from this family usually harbor a single resistance gene that is located in an interchangeable cassette-like structure. A recent study on the classification of *S. aureus* plasmids based on the combination of the *rep* genes, placed plasmids belonging to the rep_{13} family in the plasmid group pGSA5, which harbors small plasmids carrying antibiotic-resistance determinants, such as the *cat* gene for chloramphenicol resistance [23]. The type 2 *smr* cassette-like structure of pSM52 presents 100% homology to the structures present on the *S. aureus* pKH8 and on the coagulase-negative staphylococci plasmids pSK108 and pPI-2. However, Rep52 showed very high homology to Rep proteins of plasmids

carrying the *cat* gene. On the other hand, homology with the Rep proteins of other *smr*-carrying plasmids, including the above mentioned pKH8, pSK108 and pI-2 was significantly lower, suggesting that at a given time point an exchange might have occurred between the *smr* cassette-like structure carried by one of these plasmids and the *cat* gene cassette located on a chloramphenicol-resistance plasmid or transposon.

Besides a complete copy of *smr*, the *smr* cassette-like structure of pSM52 carried also a partial duplicate of this gene, Δ*smr*, coding for 59 amino acid protein of which only the first 32 residues are identical to Smr. It has been previously shown that removal of the last 19 residues of Smr would render this pump non-functional [20]. Thus, the truncated form of Smr coded by pSM52 is most probably non-functional. Furthermore, the levels of resistance presented by the strains carrying *smr* and *Δsmr*, SM52 and RN4220:pSM52, are similar to the ones presented by other strains carrying a single copy of the *smr* gene [4, 22], further supporting that ΔSmr is not contributing to the biocide resistance phenotype.

Plasmid pSM52 and, consequently the *smr* gene, were correlated to the low-level resistance to quaternary ammonium compounds presented by the clinical MRSA strain SM52, namely cetrimide, benzalkonium chloride and cetylpyridium chloride, which are commonly found in biocide formulations used in the hospitals and in the community. The presence of *smr* could also be linked to an increase of resistance towards dyes, such as ethidium bromide and berberine. The determination of MICs in the presence of EIs and the fluorometric assays provided data supporting that this resistance towards biocides and dyes is driven by efflux via Smr. Strains carrying *smr*, SM52 and RN4220:pSM52, presented higher MIC values for biocides than the susceptible RN4220, and these same MICs could be reduced by EIs to the levels presented by RN4220. In addition, a prompt ethidium bromide efflux activity was detected by fluorometry for strains SM52 and RN4220:pSM52, which was much less evident for RN4220. On the other hand, no relation was observed between Smr and resistance to phosphonium derivatives, such as tetraphenylphosphonium bromide or resistance to antibiotics.

Data from an earlier work on the MRSA isolate SM52 demonstrated the inducibility of the *smr* gene by ethidium bromide but not by ciprofloxacin, in agreement

with the substrate specificity of this pump [7]. The combination of the data from these studies indicates that Smr is responsible for the biocide and dye resistance phenotype of strain SM52 and that the inducibility of this pump by such compounds may be a decisive factor for its dissemination among *S. aureus* strains and possibly other staphylococci by mobile elements such as pSM52.

4.5. Conclusions

Plasmid pSM52 is a new RCR plasmid, member of the rep_{13} family and of the S. *aureus* plasmid group pGSA5, which carries the Smr determinant, responsible for the low-level resistance to quaternary ammonium compounds found in the clinical MRSA strain SM52. This plasmid appears to result from an arrangement between antibiotic/antiseptic-resistance plasmids present in different staphylococci.

The finding of a plasmid in a clinical MRSA strain harboring a single antiseptic resistance efflux pump gene, *smr*, strengthens the importance of plasmid-encoded efflux pumps in the maintenance of *S. aureus* strains in the hospital environment. The general use of antiseptics and disinfectants for the prevention and control of both nosocomial and community infections could potentiate the dissemination of isolates carrying antiseptic resistance plasmids.

4.6. References

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CHAPTER 5

Resistance to fluoroquinolones mediated by efflux

5. Resistance to fluoroquinolones mediated by efflux

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Summary

Antimicrobial resistance mediated by efflux systems is still poorly characterized in *Staphylococcus aureus*, despite the description of several efflux pumps for this bacterium. In this Chapter is described the use of several methodologies to characterize the efflux activity of 52 *S. aureus* isolates resistant to ciprofloxacin collected in a hospital in Lisbon, Portugal, in order to understand the role played by these systems in the resistance to fluoroquinolones. Augmented efflux activity was detected in 12 out of the 52 isolates and correlated with increased resistance to fluoroquinolones. Addition of efflux inhibitors did not result in the full reversion of the fluoroquinolone resistance phenotype, yet it implied a significant decrease in the resistance levels, regardless of the type(s) of mutation(s) found in the quinolone-resistance determining region of *grlA* and *gyrA* genes, which accounted for the remaining resistance that was not efflux-mediated. Expression analysis of the genes coding for the main efflux pumps revealed increased expression only in the presence of inducing agents, ciprofloxacin or ethidium bromide. Moreover, it showed that not only different substrates can trigger expression of different EP genes, but also that the same substrate can promote a variable response, according to its concentration. It was also found that isolates belonging to the same clonal type showed different responses towards drug exposure, thus evidencing that highly related clinical isolates may diverge in the efflux-mediated response to noxious agents. The data gathered by real-time fluorometric and RT-qPCR assays suggests that *S. aureus* clinical isolates may be primed to efflux antimicrobial compounds.

The results obtained in this study do not exclude the importance of mutations in resistance to fluoroquinolones in *S. aureus*, yet they underline the contribution of efflux systems for the emergence of high-level resistance. All together, they highlight the potential role played by efflux systems in the development of resistance to fluoroquinolones in clinical isolates of *S. aureus*.

5.1. Introduction

Staphylococcus aureus infections, particularly those caused by methicillin-resistant *S. aureus* (MRSA), pose serious therapeutic difficulties and are a major concern in both the nosocomial and community settings. The use of fluoroquinolones for the effective treatment of these infections is impaired by the swift emergence of fluoroquinolone resistance, a trait widely spread among clinical MRSA strains [8, 34].

Fluoroquinolone resistance in *S. aureus* has been mainly attributed to mutations occurring in the quinolone-resistance determining region (QRDR) of GrlA/GrlB (topoisomerase IV, encoded by genes *grlA/grlB*) and GyrA/GyrB (DNA gyrase, encoded by genes *gyrA/gyrB*); which decrease their affinity to the drug [7, 22, 29]. However, fluoroquinolone resistance can also be mediated by drug efflux, a mechanism that is less well characterized [10].

To date, several efflux pumps (EPs) have been described for *S. aureus*, including the chromosomally encoded NorA, NorB, NorC, MdeA, MepA, SepA and SdrM, as well as the plasmid-encoded QacA/B, QacG, QacH, QacJ and Smr [26]. Whereas these efflux pumps show different substrate specificity, most of them are capable of extruding compounds of different chemical classes. These features reveal the potential role of EPs in providing the cell with the means to develop a multidrug resistance (MDR) phenotype and consequently survive in hostile environments.

A variety of methods have been used to identify active efflux systems in bacteria, such as the use of radiolabelled substrates, fluorometric assays or the determination of the minimum inhibitory concentration (MIC) for different substrates in the presence of compounds known to modulate the activity of efflux pumps (usually described as efflux inhibitors, EIs) [6, 15, 35]. This work aimed to assess and characterize the presence of active efflux systems in clinical isolates of *S. aureus* using several methodologies and to understand their role in the development of resistance to fluoroquinolones by *S. aureus* in the clinical setting, since fluoroquinolones are considered substrates of the majority of the pumps encoded by the *S. aureus* chromosome [26].

5.2. Material and Methods

Bacterial isolates. Reference strains. *S. aureus* strain ATCC25923, a clinical isolate collected at Seattle in 1945 and ATCC25923$_{EtBr}$ [5], belonging to the culture collection of the Grupo de Micobactérias, Unidade de Microbiologia Médica, Instituto de Higiene e Medicina Tropical (IHMT/UNL), were used as controls. **Clinical strains.** A collection of 52 *S. aureus* was studied, comprising all the ciprofloxacin resistant clinical isolates sampled at the Bacteriology Laboratory of a 1,300-bed teaching hospital (Lisbon, Portugal), from December 2006 to March 2007. These corresponded to 49 MRSA and 3 MSSA, isolated from single patients and different biological products. All isolates were tested for identification and antibiotic susceptibility by the automated system WalkAway® (Dade Behring™) and selected on the basis of their resistance to ciprofloxacin.

Growth conditions. Strains were grown in tryptone soy broth (TSB) at 37ºC with shaking or in tryptone soy agar (TSA) (Oxoid Ltd., Basingstoke, UK). Strain ATCC25923$_{EtBr}$ was grown in TSB or TSA supplemented with 50 mg/L of EtBr. For determination of minimum inhibitory concentrations (MICs), cultures were grown in Mueller-Hinton broth (MH, Oxoid) at 37ºC.

Antibiotics and dyes. Antibiotics in powder form were purchased from different sources, as follows: nalidixic acid (Sigma-Aldrich, St. Louis, MO, USA); norfloxacin (ICN Biomedicals Inc., Ohio, USA); ciprofloxacin (Fluka Chemie GmbH, Buchs, Switzerland). EtBr was acquired in powder form from Sigma (Madrid, Spain).

Efflux inhibitors (EIs). Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), thioridazine (TZ), chlorpromazine (CPZ), verapamil (VER) and reserpine (RES) were purchased from Sigma. Solutions of TZ, CPZ and VER were prepared in desionized water; RES was prepared in dimethylsulfoxide (DMSO) and CCCP in 50% methanol (v/v). All solutions were prepared on the day of the experiment and kept protected from light.

EtBr-agar Cartwheel (EtBrCW) Method. This simple method tests the presence of active efflux systems [19, 21, 32], being an update of the already described, EtBr-agar screening method [20, 32]. It provides information on the capacity of each isolate to

extrude EtBr from the cells by efflux pumps, on the basis of the fluorescence emitted by cultures swabbed in EtBr-containing agar plates. Briefly, each culture was swabbed onto TSA plates containing EtBr concentrations ranging from 0.5 to 2.5 mg/L (0.5 mg/L increments). *S. aureus* cultures ATCC25923 and ATCC25923$_{EtBr}$ were used as negative and positive controls for efflux activity, respectively [5]. The plates were incubated at 37ºC during 16 h, after which the minimum concentration of EtBr associated with the bacterial mass that produced fluorescence under UV light was recorded in a Gel-Doc XR apparatus (Bio-Rad, Hercules, CA, USA). Isolates showing fluorescence at lower EtBr concentrations have potentially less active efflux systems than isolates for which fluorescence is only detected at higher concentrations of EtBr [19, 21, 32]. Isolates showing emission of fluorescence only at the maximum concentration of EtBr tested (2.5 mg/L) were considered to have potential active efflux systems.

Drug susceptibility testing. Antibiotics and EtBr. MICs for antibiotics were determined by the two-fold broth microdilution method [4]. Results were evaluated according to the CLSI breakpoints [4], except for nalidixic acid, for which there are no defined breakpoints. MICs for EtBr were also determined using the two-fold broth microdilution method. After an 18 h incubation period at 37ºC, the MIC values were recorded, corresponding to the lowest concentration of EtBr that presented no visible growth. All MICs were determined in triplicate. **Efflux inhibitors (EIs).** Each EI employed in this study was evaluated for its ability to reduce or reverse resistance to given antibiotics or EtBr, both of which are characteristics that define the agent as an inhibitor of efflux pump activity [18]. The evaluation of an agent for EI activity was conducted in medium containing varying concentrations of the antibiotic or EtBr and a bacterial inoculum corresponding to the one used for MIC determination. Parallel cultures were tested in media containing no EI and EI (at sub-lethal concentrations, see below) plus varying concentrations of the compound to be tested. The cultures were incubated for 18 h and growth evaluated visually. An EI was considered to have an inhibitory effect when a decrease of at least four-fold in the MIC was observed in the presence of that EI, relatively to the original MIC [6]. MICs of each EI were determined by the two-fold broth microdilution method, as described above. The final concentrations of the EIs used, which correspond to half, or below, the MICs

determined for each EI, were: TZ (12.5 mg/L); CPZ (25 mg/L); VER (200 mg/L); RES (20 mg/L) and CCCP (0.25 mg/L). All assays were performed in triplicate.

Semi-automated fluorometric method. This method allows the real-time fluorometric detection of the accumulation of a given efflux pump substrate (in this case, EtBr) inside cells and its efflux, using a Rotor-Gene 3000^{TM} thermocycler, together with real-time analysis software (Corbett Research, Sydney, Australia) [23, 31, 32]. Accumulation assays allow to assess the EtBr concentration above which detectable EtBr accumulation occurs and to select the most effective efflux inhibitor; that is the EI that promotes the highest EtBr accumulation [33]. These conditions can then be used to load bacterial cells with EtBr and follow its efflux. For the accumulation assays, the cultures were grown in TSB medium at 37ºC with shaking until they reach an optical density at 600 nm (OD_{600nm}) of 0.6. To prepare the cellular suspension, the cells were collected by centrifugation at 13,000 rpm for 3 min and the pellet washed twice with a 1X Phosphate Buffered Saline (PBS) solution. The OD_{600nm} of the cellular suspension was then adjusted to 0.6 in 1X PBS. To determine the EtBr concentration where there is detectable accumulation, several assays were prepared in 0.1 mL (final volume) containing 0.05 mL of the cellular suspension (final OD_{600nm} of 0.3) and 0.05 mL of 2X EtBr stock solutions (final concentrations of 0.25, 0.5, 1, 2, 3, 4 and 5 mg/L). To determine the most effective EI, assays were prepared in a final volume of 0.1 mL containing 0.05 mL of the cellular suspension (final OD_{600nm} 0.3) and 0.05 mL of a solution containing 2X the EtBr concentration previously selected and 2X the EI concentration to be tested (final concentrations of TZ: 12.5 mg/L, CPZ: 25 mg/L, VER: 200 mg/L, RES: 20 mg/L). All assays included control tubes containing only the isolate (0.05 mL of cellular suspension at OD_{600nm} of 0.6 plus 0.05 mL of 1X PBS) and only the EtBr concentration to be tested (0.05 mL of 2X EtBr stock solution plus 0.05 mL of 1X PBS). The assays were then run in a Rotor-Gene 3000^{TM} at 37ºC, and the fluorescence of EtBr was measured (530/585 nm) at the end of every cycle of 60 sec, for a total period of 60 min. For the efflux assays, EtBr-loaded cells were prepared by incubating a cellular suspension with an OD_{600nm} of 0.3 with either 0.25 or 1 mg/L EtBr for EtBrCW-negative or positive cultures, respectively and 200 mg/L VER at 25ºC for 60 min. After EtBr accumulation, cells were collected by centrifugation and re-suspended in 1X PBS to an OD_{600nm} of 0.6. Several parallel assays were then run in 0.1

mL final volume corresponding to 0.05 mL of the EtBr loaded cells (final OD_{600nm} of 0.3) incubated with 0.05 mL of (1) PBS 1X only; (2) glucose 0.8% only (final concentration of 0.4%); (3) 2X VER only (final concentration of 200 mg/L); (4) glucose 0.8% (final concentration of 0.4%) plus 2X VER (final concentration of 200 mg/L). These efflux assays were conducted in the Rotor–Gene 3000™ at 37ºC, and the fluorescence of EtBr was measured (530/585 nm) at the end of every cycle of 10 sec, for a total period of 10 min. The raw data obtained was then normalized against data obtained from non-effluxing cells (cells from the control tube with only 200 mg/L VER), at each point, considering that these correspond to the maximum fluorescence values that can be obtained during the assay. The relative fluorescence thus corresponds to the ratio of fluorescence that remains per unit of time, relatively to the EtBr-loaded cells.

Macrorestriction analysis. Isolates were typed by pulsed-field gel electrophoresis (PFGE) analysis, using well-established protocols. Briefly, agarose disks containing intact chromosomal DNA were prepared as previously described [3] and restricted with SmaI (New England Biolabs, Ipswich, MA, USA), according to the manufacturer's recommendations. Restriction fragments were then resolved by PFGE, which was carried out in a contour-clamped homogeneous electric field apparatus (CHEF-DRIII, Bio-Rad), as previously described [3]. Lambda ladder DNA (New England Biolabs) was used as molecular weight marker. PFGE types were defined according to the criteria of Tenover *et al*. [30].

Preparation of chromosomal DNA. Genomic DNA was extracted with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), with an additional step of 30 min digestion with lysostaphin (Sigma) (200 mg/L) prior to extraction.

Preparation of plasmid DNA. The QIAprep Spin Miniprep kit (QIAGEN) was used, with the following modification: prior to extraction, cells were incubated with lysostaphin (35 mg/L) at 37ºC for 90 min, as previously described [1].

Screening of mutations in *grlA* and *gyrA* genes. Internal fragments comprising the QRDR of *grlA* and *gyrA* genes were amplified using the primers described in Table 5.1. The reaction mixture (50 μL) contained 2.5 U of Taq Polymerase (Fermentas Inc., Ontario, Canada), 1X Taq buffer (Fermentas); 25 pmol of each primer; 0.2 mM of

dNTP and 1.75 mM of $MgCl_2$. The PCR reactions were conducted in a thermocycler Mastercycler personal 5332 (Eppendorf AG, Hamburg, Germany). The amplification conditions were as follows: DNA was denatured at 94ºC for 4 min, followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 50ºC for 30 sec and extension at 72ºC for 1 min, followed by a step of final extension at 72ºC for 5 min. Amplification products were purified and sequenced in both strands using the same set of primers. Sequences were analyzed and aligned using the freeware programs BioEdit and ClustalW, respectively.

PCR amplification of efflux pump genes. DNA fragments internal to five chromosomal and two plasmid encoded efflux pump genes were separately amplified by PCR, using the primers described in Table 5.1. Reaction mixtures were prepared as described above. Amplification conditions were as follows: DNA was denatured at 94ºC for 4 min, followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 45ºC (*norA*) or 53ºC (*norB, norC, mdeA, mepA*) for 30 sec and extension at 72ºC for 1 min, followed by a step of final extension at 72ºC for 5 min. The PCR reactions for genes *qacA/B* and *smr* were conducted under the following conditions: DNA was denatured at 95ºC for 1 min, followed by 30 cycles of denaturation at 95ºC each for 1 min, annealing at 40ºC (*qacA/B*) or 48ºC (*smr*) for 1 min and extension at 72ºC for 1 min, followed by a step of final extension at 72ºC for 5 min. The amplification products were visualized by 1% agarose gel electrophoresis.

RNA extraction. For RNA extraction, strains were cultured in TSB media containing ciprofloxacin or EtBr, at ½ their MIC for each strain or in drug-free TSB, and grown until an OD_{600nm} of 0.6. Total RNA was extracted with the RNeasy Mini Kit (QIAGEN), following the manufacturer's instructions. Before extraction of total RNA, cultures were treated with the RNAprotect bacterial reagent (QIAGEN). Contaminating DNA was removed with RNase-free DNase (QIAGEN) by a two hours on-column digestion at room temperature.

RT-qPCR protocol. Quantitative RT-PCR (RT-qPCR) was performed using the QuantiTect SYBR Green RT-PCR Kit (QIAGEN). The primers used in these assays are described in Table 5.1. The relative quantity of mRNA corresponding to genes *norA, norB, norC, mepA, mdeA* and *smr* was determined by the comparative threshold cycle

(C_T) method [17] in a Rotor-Gene 3000™ thermocycler with real-time analysis software. Relative expression of the efflux pump genes was assessed by two approaches: (i) comparison of the relative quantity of the respective mRNA in the *S. aureus* isolates to the one present in a reference strain, ATCC25923; (ii) comparison of the relative quantity of the respective mRNA in the presence of ciprofloxacin or EtBr (at ½ the MIC) to the drug-free condition. For each strain, three assays were conducted, corresponding to three independent total RNA extractions. Negative controls and genomic DNA contamination controls were included. 16S rDNA was used as reference. Genes showing increased expression of at least four-fold, when compared to the drug-free condition, were considered to be overexpressed [6].

Table 5.1. Primers used in this study.

Primer[a]	Sequence (5'-3')	Amplicon Size (bp)	Reference
QacA/B_Fw	GCTGCATTTATGACAATGTTTG	628	[1]
QacA/B_Rv	AATCCCACCTACTAAAGCAG		
Smr_Fw	ATAAGTACTGAAGTTATTGGAAGT	285	[2]
Smr_Rv	TTCCGAAAATGTTTAACGAAACTA		
NorA_Fw	TTCACCAAGCCATCAAAAAG	620	[28]
NorA_Rv	CTTGCCTTTCTCCAGCAATA		[5]
NorA_Fw	TTCACCAAGCCATCAAAAAG	95	[28]
NorA_RT(Rv)	CCATAAATCCACCAATCCC		This study
NorB_Fw	AGCGCGTTGTCTATCTTTCC	213	[5]
NorB_Rv	GCAGGTGGTCTTGCTGATAA		
NorC_Fw	AATGGGTTCTAAGCGACCAA	216	[5]
NorC_Rv	ATACCTGAAGCAACGCCAAC		
MepA_Fw	ATGTTGCTGCTGCTCTGTTC	718	[5]
MepA_Rv	TCAACTGTCAAACGATCACG		
MepA_RT(Fw)	TGCTGCTGCTCTGTTCTTTA	198	[5]
MepA_RT(Rv)	GCGAAGTTTCCATAATGTGC		

[a]The primers used in the RT-qPCR experiments are indicated by the RT label. Fw: forward; Rv: reverse. For *norB*, *norC* and *smr*, the same set of primers was used for both PCR and RT-qPCR, as well as the primer NorA_Fw.

Table 5.1. ***(Cont.)*** **Primers used in this study.**

Primer[a]	Sequence (5'-3')	Amplicon Size (bp)	Reference
MdeA_Fw	AACGCGATACCAACCATTC	677	[5]
MdeA_Rv	TTAGCACCAGCTATTGGACCT		
MdeA_RT(Fw)	GTTTATGCGATTCGAATGGTTGGT	155	[13]
MdeA_RT(Rv)	AATTAATGCAGCTGTTCCGATAGA		
16S_27f	AGAGTTTGATCMTGGCTCAG	492	[16]
16S_519r	GWATTACCGCGGCKGCTG		
GrlA_Fw	TGCCAGATGTTCGTGATGGT	339	[24]
GrlA_Rv	TGGAATGAAAGAAACTGTCTC		
GyrA_Fw	TCGTGCATTGCCAGATGTTCG	394	[24]
GyrA_Rv	TCGAGCAGGTAAGACTGACGG		

[a]The primers used in the RT-qPCR experiments are indicated by the RT label. Fw: forward; Rv: reverse.

5.3. Results

5.3.1. Detection of active efflux systems by the Ethidium Bromide (EtBr)-agar Cartwheel (EtBrCW) Method

For this study, we selected all the *S. aureus* isolates presenting resistance towards ciprofloxacin received by the Bacteriology Laboratory of one of the largest hospitals in Portugal during a four months period. These corresponded to a collection of 52 *S. aureus* isolates.

Efflux activity amongst these 52 ciprofloxacin resistant isolates was assessed by means of a fast and practical test, the Ethidum Bromide-agar Cartwheel (EtBrCW) Method that provides information on the capacity of each isolate to extrude EtBr from the cells by efflux, on the basis of the fluorescence emitted by cultures swabbed in EtBr-containing agar plates. Those cultures showing fluorescence at lower EtBr concentrations have potentially less active efflux systems than those for which fluorescence is only detected at higher concentrations of EtBr [19, 21]. The application of this method allowed the selection of 12 *S. aureus* isolates showing increased EtBr efflux activity when compared to the non-effluxing control strain ATCC25923 and to

the efflux-positive control strain ATCC25923$_{EtBr}$ [5]. These 12 isolates were designated EtBrCW-positive isolates, whereas the remaining 40 isolates were considered to have no or intermediate efflux activity and therefore designated as EtBrCW-negative isolates (Table 5.2).

Based upon these results, we continued the study by further analyzing the 12 EtBrCW-positive isolates, as well as a group of representative 13 EtBrCW-negative isolates, as controls.

5.3.2. Real-time assessment of efflux activity

In order to characterize the efflux activity of the cells, we used a semi-automated fluorometric method previously developed by our group [33], which allows monitoring, on a real-time basis, the accumulation of EtBr inside the bacterial cells, followed by its efflux.

The first step of this technique is to establish the ideal conditions for EtBr accumulation inside the cells. Thus, assays were initially performed to determine the EtBr concentration above which there is detectable accumulation and to select the most effective efflux inhibitor; that is the EI that promotes the highest EtBr accumulation. The EtBr accumulation assays showed that the two groups of isolates previously established by the EtBrCW Method differed with respect to their capacity to accumulate EtBr, with EtBrCW-negative isolates retaining more EtBr than the EtBrCW-positive isolates (Figure 5.1-A). The same result was observed for the reference strain ATCC25923. These differences were reflected in the minimum EtBr concentration required for detectable accumulation, which was higher for the EtBrCW-positive isolates. The accumulation assays performed in the presence of several EIs showed that verapamil was the most effective in promoting accumulation of EtBr, for either EtBrCW-positive isolates, EtBrCW-negative isolates or the reference strain (Figure 5.1-B).

The conditions established by the accumulation assays were then used to load cells with EtBr and perform efflux assays. The assessment of EtBr efflux on a real-time

basis (during a 10 min frame) detected a considerable difference between EtBrCW-positive isolates, which showed a pronounced efflux pump activity, with a prompt and significant decrease in fluorescence and the EtBrCW-negative isolates, that showed only basal efflux pump activity, similar to the one presented by the reference strain (Figure 5.1-C). These results confirm the presence of increased efflux activity in the EtBrCW-positive isolates relatively to the EtBrCW-negative isolates.

5.3.3. Effect of efflux inhibitors on MICs of fluoroquinolones and EtBr

As expected, since all clinical isolates were selected on the basis of resistance to ciprofloxacin, they all presented high MIC values for fluoroquinolones. Nevertheless, the majority of the EtBrCW-positive isolates displayed higher MIC values for the fluoroquinolones tested and EtBr, whilst the EtBrCW-negative isolates presented significantly lower values, although some overlap exists between the two sets of MIC values (Table 5.2). The EIs reduced the MIC values for fluoroquinolones and EtBr of the EtBrCW-positive isolates to the values presented by the EtBrCW-negative isolates, confirming the presence of an active efflux component in those isolates (Table 5.2). The EIs thioridazine (TZ) and chlorpromazine (CPZ) were the most effective in reducing the MIC values. Verapamil (VER) and reserpine (RES) showed a smaller or absent inhibitory effect, while carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) showed no effect on the MIC values for the compounds tested (data not shown). However, no full reversion of the fluoroquinolone resistance phenotype was obtained with any of the EIs tested, suggesting the contribution of other mechanisms to this resistance, namely, mutations in the target genes.

Table 5.2. Genotypic and phenotypic characterization of *S. aureus* clinical isolates.

Isolate[a]	PFGE pattern	QRDR mutations[b]		MIC (mg/L)[c]											
				EtBr			CIP			NOR			NAL		
		GrlA	GyrA	No EI	+ TZ	+ CPZ	No EI	+ TZ	+ CPZ	No EI	+ TZ	+ CPZ	No EI	+ TZ	+ CPZ
ATCC25923	-	WT	WT	6.25	**0.75**	**0.75**	0.25	0.125	0.125	0.5	**0.125**	**0.125**	64	n.d.	n.d.
ATCC25923$_{EtBr}$	-	WT	WT	200	**25**	**12.5**	1	**0.25**	**0.25**	2	**0.25**	**0.25**	64	n.d.	n.d.
SM1	A2	S80Y/E84G	S84L	16	**4**	**4**	128	**32**	64	512	**128**	256	256	**64**	**64**
SM10	A4	S80Y/E84G	S84L	16	**2**	**4**	128	64	64	512	**128**	**128**	128	64	64
SM14	A3	S80Y/E84G	S84L	16	**4**	**4**	256	**32**	128	1024	**128**	**256**	256	**64**	**64**
SM17	A4	S80Y/E84G	S84L	16	**4**	**4**	256	**64**	**64**	1024	**256**	512	256	**64**	**64**
SM25	A1	S80Y/E84G	S84L	8	**2**	4	128	**32**	64	512	**64**	**128**	256	**32**	**64**
SM27	A4	S80Y/E84G	S84L	16	**4**	**4**	256	**32**	**64**	512	**128**	256	256	**64**	**64**
SM43	A1	S80Y/E84G	S84L	16	**2**	**4**	128	64	64	512	**128**	**128**	512	256	**64**
SM46	A1	S80Y/E84G	S84L	16	**4**	**4**	128	64	64	512	**128**	256	128	64	64
SM47	A1	S80Y/E84G	S84L	8	**2**	4	256	**32**	**64**	512	**128**	256	256	**64**	**64**
SM48	A1	S80Y/E84G	S84L	8	4	4	256	**32**	**64**	512	**128**	256	256	**64**	**64**
SM50	B1	S80F/E84K	S84L	8	**1**	**2**	64	**16**	**16**	256	**32**	**64**	128	64	64
SM52	C1	S80Y	S84L	16	**1**	**2**	16	8	8	64	32	32	128	**32**	64
SM2	B2	S80F/E84K	S84L	8	**2**	**2**	32	16	16	128	**32**	**32**	64	**16**	64
SM3	E1	S80F/E84G	S84L	1	1	1	16	8	8	64	32	32	64	**16**	**16**
SM4	E2	S80F	S84L	4	2	**1**	8	8	8	64	32	32	64	32	64
SM5	E3	S80F/E84G	S84L	4	2	**1**	32	16	16	128	64	64	64	32	32
SM6	A5	S80F	E88K	4	2	**1**	16	16	16	64	32	32	64	32	32
SM7	E1	S80F	S84L	2	2	1	8	8	4	64	32	32	128	**32**	64
SM8	A5	S80F	E88K	4	2	**1**	16	8	16	128	64	64	128	**32**	64
SM12	E1	S80F	S84L	2	2	1	16	8	8	64	32	32	128	**32**	64
SM16	A6	S80F	E88K	4	2	**1**	16	16	16	128	**32**	64	64	32	64
SM22	A1	S80Y/E84G	S84L	8	4	4	128	**16**	**32**	512	**128**	**128**	64	32	64
SM34	D1	S80F/E84K	S84L	4	2	2	64	**16**	32	64	**16**	32	32	16	32
SM36	E1	S80F	S84L	4	2	2	16	8	8	64	**16**	32	128	**32**	64
SM40	E1	S80F	S84L	8	4	4	32	32	32	512	**128**	**128**	16	8	16

[a]Isolates in bold correspond to the EtBrCW-positive isolates. [b]WT: wild-type; S: serine; F: phenylalanine; E: glutamic acid; K: lysine; Y: tyrosine; L: leucine; G: glycine. [c]Values in bold-type correspond to a MIC decrease of ≥ four-fold in the presence of the efflux inhibitor (EI) in comparison to the values with no EI [6]. The concentration of each EI used is defined in the Material Methods section. EtBr: ethidium bromide; CIP: ciprofloxacin; NOR: norfloxacin; NAL: nalidixic acid; TZ: thioridazine; CPZ: chlorpromazine; n.d.: not determined.

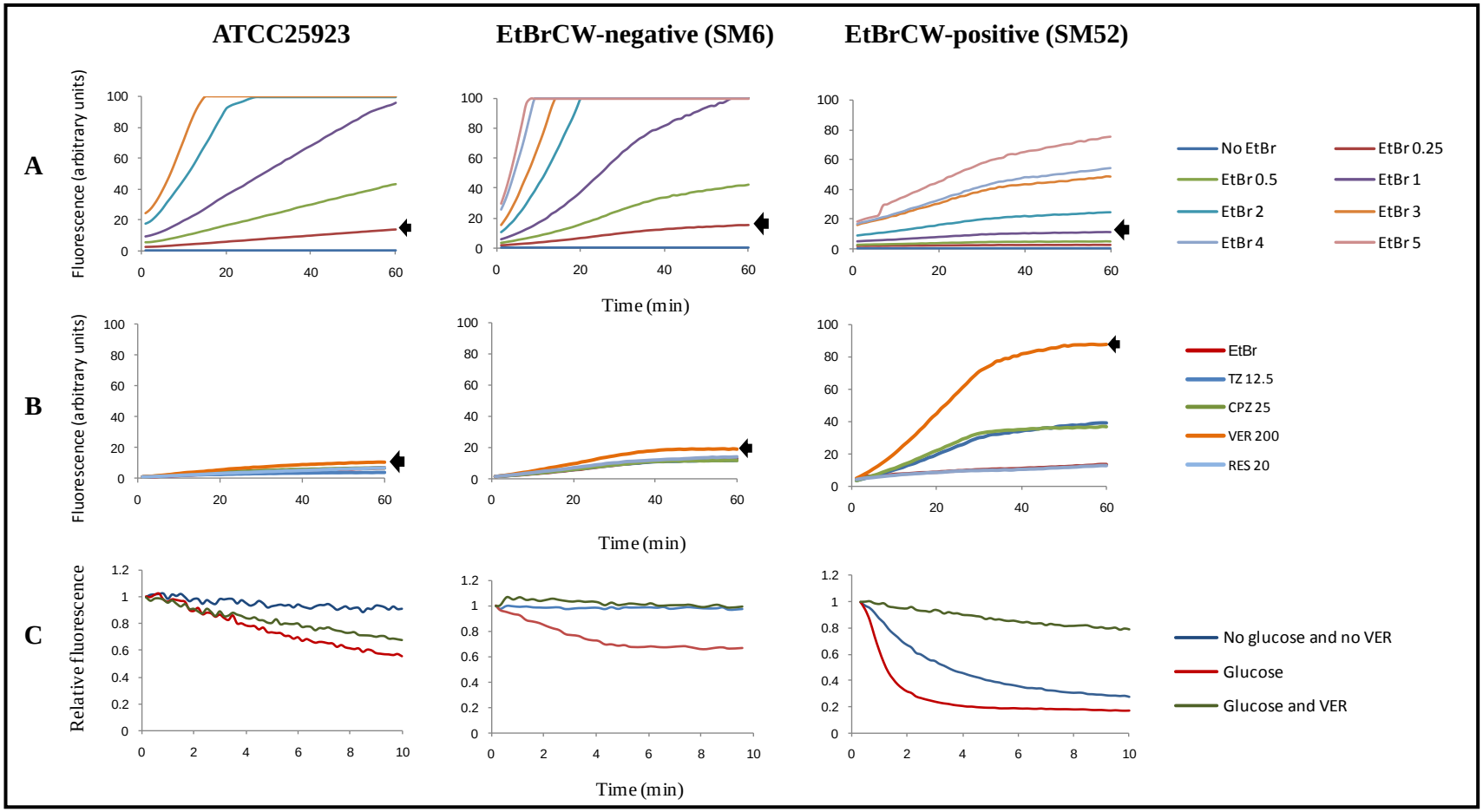

Figure 5.1. Real-time EtBr accumulation/efflux for the representative strains ATCC25923 (reference), SM6 (EtBrCW-negative) and SM52 (EtBrCW- positive). Panel A: Assessment of EtBr accumulation. The arrow indicates the EtBr accumulation at the concentration (mg/L) chosen for the subsequent assays (panels B and C). **Panel B: Assessment of EtBr accumulation in the presence of efflux inhibitors.** The EIs were tested at a sub-inhibitory concentration, namely TZ: thioridazine (12.5 mg/L); CPZ: chlorpromazine (25 mg/L); VER: verapamil (200 mg/L) and RES: reserpine (20 mg/L). The arrow indicates the EtBr accumulation in the presence of the most effective EI for each isolate. **Panel C: Assessment of EtBr efflux.** The assays were done in the presence/absence of 0.4% glucose, with or without the EI verapamil (VER) at a sub-inhibitory concentration of 200 mg/L. The data presented was normalized against the data obtained in conditions of no efflux (absence of glucose and presence of 200 mg/L of VER).

5.3.4. Screening for mutations conferring fluoroquinolone resistance

The 25 isolates representing both EtBrCW-positive and negative isolates were screened for the presence of chromosomal mutations most commonly associated with fluoroquinolone resistance in *S. aureus*, namely the ones occurring in the QRDRs of both *grlA* and *gyrA* genes [7, 11, 12, 29]. All isolates tested carried mutations in *grlA* and *gyrA* related to fluoroquinolone resistance, in six different combinations at the protein level (Table 5.2). The majority of the isolates presented a double mutation in GrlA together with a single mutation in GyrA, with 11 isolates carrying the GrlA and GyrA mutations S80Y/E84G and S84L, respectively; three isolates carrying mutations GrlA S80F/E84K and GyrA S84L; and two isolates carrying mutations GrlA S80F/E84G and GyrA S84L. The other nine isolates screened showed a single mutation in both GrlA and GyrA, in three distinct arrangements (Table 5.2).

The overall analysis of these results reveals a clear distinction between the EtBrCW-positive and the EtBrCW-negative isolates, with each group showing a relatively homogeneous profile, both in terms of efflux capacity and mutations in the genes related to fluoroquinolone resistance. In order to test if such homogeneity would be the result of clonal expansion of specific *S. aureus* clones, the isolates were then typed by macrorestriction analysis.

5.3.5. Macrorestriction analysis

The clonality of the *S. aureus* clinical isolates was assessed by pulsed-field gel electrophoresis (PFGE) analysis of SmaI macrorestriction profiles. According to the criteria of Tenover *et al.* [30], six clones were found among the entire collection. The two predominant clones, A and E, included several sub-clones and comprised 25 and 18 isolates, respectively. The remaining clones B, C, D and F, were represented by 1 to 6 isolates (representative data is presented in Table 5.2 and Figure 5.2).

Of the 12 EtBrCW-positive isolates, 10 belonged to clone A, one to clone B and one to clone C. On the other hand, the 40 EtBrCW-negative isolates included all isolates

from clone E (18 isolates) plus isolates from clone A (15), clone B (5), clones D and F (1 isolate each).

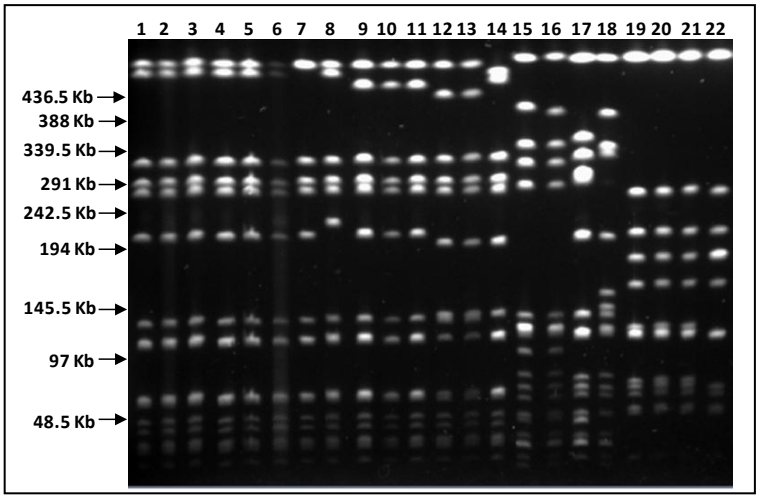

Figure 5.2. SmaI macrorestriction profiles of *S. aureus* clinical isolates. Numbers correspond to the following isolates: 1- SM43; 2- SM46; 3- SM47; 4- SM48; 5- SM22; 6- SM25; 7- SM1; 8- SM14; 9- SM10; 10- SM17; 11- SM27; 12- SM6; 13- SM8; 14- SM16; 15- SM50; 16- SM2; 17- SM52; 18- SM34; 19- SM36; 20- SM40; 21- SM3; 22- SM4. The arrows show the position and weight of the lambda ladder molecular size marker.

5.3.6. Expression analysis of *S. aureus* efflux pump genes

The presence of EP genes was assessed by PCR. All *S. aureus* isolates carried the five chromosomal genes tested (*norA*, *norB*, *norC*, *mepA* and *mdeA*) and one isolate, SM52, carried the plasmid encoded *smr* gene, whereas no isolate was found to carry the plasmid encoded *qacA/B* gene.

To assess the contribution of each individual pump to the overall efflux activity presented by each strain, ten isolates representative of each clone or sub-clone (six EtBrCW-positive and four EtBrCW-negative) plus reference strain ATCC25923 (also EtBrCW-negative), were selected for expression analysis by RT-qPCR of EP genes.

When determining the expression level of efflux pump genes for the clinical isolates in comparison to the reference strain ATCC25923 in drug-free media, no overexpression of these genes was detected (data not shown). When these clinical isolates and ATCC25923 were exposed to an efflux pump substrate, either ciprofloxacin or EtBr, at ½ their MICs, and gene expression levels determined against the respective

unexposed condition, overexpression of efflux pump genes was detected in six clinical isolates, three EtBrCW-negative and three EtBrCW-positive as well as in the reference strain itself (Table 5.3).

The majority of the isolates showed overexpression of a single efflux pump gene, most frequently, *norB* or *mdeA*. One isolate showed overexpression of two efflux pump genes (*norB/norC*) and another one overexpressed three EP genes (*norB*/*norC*/*mepA*). Overall, isolates showed to be more responsive to ciprofloxacin. The *smr* gene was found to be overexpressed only in the presence of EtBr, in accordance to the substrate specificity described in the literature for this pump [2]. These same agents had a distinct effect on ATCC25923, which showed significant overexpression of all efflux pump genes tested in the presence of EtBr, and a higher overexpression of *norB* when exposed to ciprofloxacin (Table 5.3).

The effect of drug exposure on the expression level of the efflux pump genes was further explored by increasing the ciprofloxacin concentration to ¾ the MIC. Isolates that showed EP gene overexpression with ½ the MIC of ciprofloxacin showed either an increase in that expression level or the overexpression of additional genes. For instance, EtBrCW-positive isolate SM50 overexpressing *norB*/*norC* with ½ MIC of ciprofloxacin, now showed even higher expression of *norB* (37.05 ± 18.67) and *norC* (83.98 ± 19.98) and *de novo* overexpression of *norA* (8.36 ± 4.63) and *mepA* (45.86 ± 13.86). Likewise, exposure of the EtBrCW-negative SM2 to higher ciprofloxacin concentrations resulted in increased levels of *norB* expression (4.48 ± 2.48) that was accompanied by *de novo* overexpression of *norC* (5.33 ± 0.73) and *mepA* (10.58 ± 0.73).

Table 5.2. EP gene expression analysis by RT-qPCR of representative *S. aureus* exposed to CIP or EtBr.

Gene	Overexpression levels* and no. of isolates** showing gene overexpression					
	½ CIP MIC			½ EtBr MIC		
	ATCC25923	EtBrCW- isolates (n = 4)	EtBrCW+ isolates (n = 6)	ATCC25923	EtBrCW- isolates (n = 4)	EtBrCW+ isolates (n = 6)
norA	-	- 0	- 0	4.51 ± 0.77	- 0	- 0
norB	13.80 ± 6.50	5.43 ± 2.39 **2[a,b]**	5.47 ± 0.19 **1[e]**	7.07 ± 2.78	5.33 ± 0.73 **1[a]**	- 0
norC	-	- 0	4.92 ± 0.00 **1[e]**	5.89 ± 0.71	4.99 ± 1.51 **1[a]**	- 0
mepA	-	- 0	8.59 ± 0.59 **1[f]**	3.90 ± 0.13	5.94 ± 1.02 **1[a]**	- 0
mdeA	-	4.97 ± 0.68 **1[c]**	- 0	3.96 ± 2.10	- 0	4.15 ± 1.12 **1[d]**
smr	n.a.	n.a.	- 0	n.a.	n.a.	7.66 ± 3.66 **1[f]**

Gene expression was measured in the presence of ciprofloxacin and EtBr relatively to the drug-free condition. * The results presented are expressed in terms of the mean ± standard deviation of at least three independent assays performed with independent extracted RNAs and correspond to the range of values obtained for isolates showing overexpression of that gene. **The numbers in bold correspond to the number of isolates overexpressing that gene: [a] isolate SM2; [b] SM3; [c] SM5; [d]SM25; [e] SM50; [f] SM52. Overexpression was considered for values ≥4 [6]. (-): no overexpression detected; n.a.: not applicable.

5.4. Discussion

The few studies on efflux among *S. aureus* clinical isolates use the decrease of antibiotic MICs in the presence of EIs, particularly reserpine, as indicative of efflux activity [6]. This approach is laborious and dependent on the susceptibility of the efflux system(s) to reserpine, which varies considerably [9]. More recently, Patel and colleagues have proposed the use of EtBr MICs to identify *S. aureus* effluxing strains [25]. This approach has the advantage of assessing efflux activity using a broad range efflux pump substrate, EtBr, which is pumped out by most efflux systems described for *S. aureus*, and thus, is an useful marker for the detection of efflux pump activity [21, 25, 26, 33]. Nevertheless, it is still an indirect assessment of efflux activity.

In the present study, we have applied two methods for the direct assessment of efflux activity among a collection of 52 ciprofloxacin resistant *S. aureus* clinical isolates, both also based on EtBr efflux. We first applied the EtBr-agar Cartwheel Method to select isolates with increased efflux activity. The presence of increased efflux in the 12 isolates selected was supported by the data collected from the semi-automated fluorometric method, which demonstrated that EtBrCW-positive isolates had a higher efflux activity than the EtBrCW-negative isolates. Thus, both methods proved to be adequate to assay efflux activity in *S. aureus* cells. In particular, the EtBrCW method proved to be a valuable tool for the rapid screening of efflux pump activity, allowing its application to screen large collections of clinical isolates. Furthermore, the use of a broad range efflux pump substrate such as EtBr warrants its wider application as compared to the analysis of EIs effect on MIC values, which can be severely impaired by the susceptibility of each efflux system to the EI being used and for which the mechanism of action at the cellular level remains, in most cases, to be clarified.

In addition, the semi-automated fluorometric method also allowed the characterization of this efflux activity, in terms of maximal concentration of EtBr that the cells were able to extrude without observable accumulation over a 60 min period and susceptibility toward several EIs. The results obtained clearly showed a distinct capacity of the two groups of isolates to extrude EtBr from their cells, with the EtBrCW-positive isolates being able to handle higher EtBr concentrations with no detectable accumulation. It was also observed that for both groups of isolates, EtBr extrusion/accumulation was most affected by the EI verapamil.

The efflux assays further demonstrated the higher efflux capacity of the EtBrCW-positive isolates, with a pronounced decreased of EtBr fluorescence (80%) within a 2 min interval, whereas the EtBrCW-negative isolates showed a smaller decrease of EtBr fluorescence (40%) over a 10 min interval.

These results were then complemented with MIC determination in the presence of EIs, leading to the observation that the efflux–mediated resistance is an important component of the level of fluoroquinolone resistance. In fact, not only the 12 EtBrCW-positive isolates presented higher MIC values towards the several fluoroquinolones, also these MICs decreased to levels similar to those of the EtBrCW-negative isolates in the

presence of TZ and CPZ, even for isolates sharing the same QRDR mutations (Table 5.2). Altogether, these data demonstrate that mutations in the QRDR of *grlA* and *gyrA* genes confer resistance up to a certain level (8-32 mg/L for ciprofloxacin), above which resistance is mainly efflux-driven. This implies that although the inhibition of the efflux component by EIs does not bring resistance down to the susceptibility level, it promotes a significant decrease in this resistance.

In the MIC assays TZ and CPZ were the two EIs with the highest effect, whereas in the fluorometric assay, EtBr extrusion/accumulation was most affected by verapamil. This should reflect differences in the mechanism of action of each molecule, as well as to the characteristics of each assay. We have recently observed the same type of results with isolates of *Mycobacterium smegmatis* [27]. The absence of efflux inhibitory effect of CCCP at sub-MIC concentrations for *S. aureus* strains has been discussed in a previous study [5].

For the analysis of gene expression, we first compared our clinical isolates to a fully-antibiotic susceptible reference strain, *S. aureus* ATCC25923, following the rationale of previous studies, [6, 14, 25]. However, in contrast to these earlier studies, no EP gene was found to be overexpressed. Consequentially, we explored the effect of exposing the isolates to ½ the MIC of the antimicrobial compounds used previously as selective markers, ciprofloxacin and EtBr, using the isolates grown in a drug-free condition as a reference for determining the gene expression level. Using this approach, we were able to detect overexpression of EP genes, albeit at levels lower than the ranges described in literature [6, 14, 25]. These differences could, in some extent, reflect the different approaches used, including the use of a different reference strain for gene expression assays. Nevertheless, the different methodological approaches do not explain all the results and since EtBrCW-positive isolates showed a strong involvement of efflux in the resistance phenotype, the absence of high levels of efflux pump genes expression suggests that the isolates could be already primed to respond to these noxious compounds. Clinical isolates are under a constant pressure by antimicrobial compounds, such as antibiotics and biocides. Since the expression of efflux pumps provides the cell with the means to cope with these compounds, it could be expected that those clinical isolates already have in their cell membrane the necessary number of efflux pump proteins, thus, increases in efflux pump genes expression may have already

taken place. Also, no significant differentiation could be established between EtBrCW-positive and EtBrCW-negative isolates at the level of individual EP gene expression (Table 5.3). On the other hand, ATCC25923, which showed only basal efflux activity on the fluorometric assay, responded to drug pressure in a completely different manner, showing a significant overexpression of all efflux pump genes tested in the presence of EtBr and the highest expression level of *norB* following exposure to ciprofloxacin (Table 5.3). The distinct behavior observed for the clinical isolates as compared to the antibiotic fully susceptible reference strain further support the hypothesis that the clinical strains are primed to efflux noxious substances.

Increasing the concentration of ciprofloxacin to ¾ of the MIC augmented the expression rate of the already overexpressed genes with the additional overexpression of other efflux pump genes. These results show a clear concentration level above which there is an inducement of expression of the same or additional efflux pump genes. This response could reflect the involvement of these genes in a global stress response regulon, or simply be the result of a substrate-responsive regulation. Future work should clarify this aspect.

A previous study described the predominance of *norB* overexpression among a collection of *S. aureus* bloodstream isolates. For this collection, when a single efflux pump gene was overexpressed, it corresponded mostly to *norA*, whereas *norB* and *norC* were prevalent when two or more efflux pump genes were overexpressed [6]. In our work, amongst the clinical isolates that overexpressed efflux pump genes, four showed overexpression of a single gene, either *norB*, *mdeA* or *mepA*. Only two isolates showed overexpression of more than one efflux pump gene. Remarkably, *norA* was the only gene for which no overexpression was detected among the clinical isolates, suggesting that other efflux pumps can have a more relevant role in the resistance to fluoroquinolones and EtBr in *S. aureus* than the one attributed to date. Nevertheless, exposure of ATCC25923 to EtBr, resulted in the overexpression of all efflux pump genes tested, including *norA*. This result does not oppose to our previous finding that the prolonged exposure of this strain to increasing concentrations of EtBr resulted in high overexpression of solely *norA* [5], inasmuch as it strengthens the premise that exposure of the same strain to a given drug over different ranges of concentrations and/or time may result in the activation of different efflux systems. Our data also

revealed that the same clinical isolate can respond differently at the gene expression level, to the presence of two inducers, ciprofloxacin and EtBr, both common substrates of the main multidrug efflux pumps in *S. aureus*. For example, the EtBrCW-negative isolate SM2 exposed to ciprofloxacin showed only *norB* overexpression, whilst in the presence of EtBr, it overexpressed *norB, norC* and *mepA*. In the particular case of strain SM52, the plasmid encoded Smr pump was only overexpressed upon exposure to EtBr, whereas when challenged with ciprofloxacin, the strain responded with the overexpression of *mepA*. Our data also demonstrates that isolates from the same clone, as defined by PFGE, can have distinct levels of efflux activity and respond to the same agent through the activation of different efflux pumps (cf Tables 5.2 and 5.3).

5.5. Conclusions

The rationale and methodologies applied in this study showed that efflux activity is an important component of the resistance to fluoroquinolones and other compounds in clinical isolates of *S. aureus*. We demonstrated that not only different substrates can trigger different pumps, but also that the same substrate can promote a variable response, according to its concentration, thus strengthening the crucial role played by efflux pumps in the survival of *S. aureus* clinical isolates in healthcare settings. Additionally, our study underlines the importance of using new molecular approaches to fully understand the function that each individual efflux pump undertakes in the bacterial cell response to antimicrobial compounds.

In particular, although specific clones could be found among either EtBrCW-positive or EtBrCW-negative bacteria, isolates belonging to the same clonal type showed different responses towards drug exposure, thus evidencing that highly related clinical isolates, sharing the same genetic background, may diverge in the efflux-mediated response to noxious compounds. The data gathered by the semi-automated fluorometric method together with the results from the RT-qPCR assays, sustain the hypothesis that *S. aureus* clinical isolates may be primed to efflux antimicrobial compounds. Therefore, the lack of a marked response to the induction of efflux pump genes expression may be explained by the higher efflux capacity already present in all

the clinical isolates tested, when compared to the naive reference strain *S. aureus* ATCC25923.

Altogether, the results presented in this study show the potential role played by efflux systems in the development of resistance to fluoroquinolones in hospitals and the contribution of the several *S. aureus* efflux systems to this resistance.

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CHAPTER 6

Resistance to antimicrobial compounds mediated by efflux

6. Resistance to antimicrobial compounds mediated by efflux

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Summary

Resistance mediated by efflux has been recognized in *Staphylococcus aureus* in the last few decades, although its clinical relevance has only been recognized recently. The existence of only a few studies on the individual and overall contribution of efflux to resistance phenotypes associated with the need of well-established methods to assess efflux activity in clinical isolates contributes greatly to the lack of solid knowledge of this mechanism in *S. aureus*. In this chapter it is presented work developed to provide information on approaches useful to the assessment and characterization of efflux activity, as well as contributing to our understanding of the role of efflux to phenotypes of antibiotic resistance and biocide tolerance in *S. aureus* clinical isolates. The results described show that efflux is an important contributor to fluoroquinolone resistance in *S. aureus* and suggest it as a major mechanism in the early stages of resistance development. It is also shown that efflux plays an important role on the reduced susceptibility to biocides in *S. aureus*, strengthening the importance of this long neglected resistance mechanism to the persistence and proliferation of antibiotic/biocide-resistant *S. aureus* in the hospital environment.

6.1. Introduction

Efflux pumps are membrane proteins that have the function of detoxifying cells by expelling noxious molecules [29]. The extrusion of antimicrobial compounds, such as antibiotics and biocides, is considered to be an "accidental function" of such efflux systems [31, 32]. Nevertheless, efflux-mediated resistance towards antimicrobial compounds is increasingly recognized as an important resistance mechanism in bacteria [30]. Efflux pumps present different substrate specificities; some are specific to an antibiotic or a class of antibiotics, whereas multidrug efflux pumps, as the name implies, have the capacity to extrude more than one class of antibiotics and/or other antimicrobial compounds [33]. These latter efflux systems are of foremost relevance, since they can bestow the bacterial cell with a phenotype of resistance to multiple drugs in

addition to promoting cross-resistance between antibiotics and other antimicrobial compounds usually used to prevent and control healthcare associated infections [30].

In *Staphylococcus aureus*, several specific efflux pumps have been associated with resistance to antibiotics, such as tetracycline (Tet(K), Tet(L)) and macrolides (Mef(A), Msr(A)) [33]. Also, several multidrug efflux pumps have been described that are associated with resistance to antibiotics (e.g., fluoroquinolones) and to biocides, such as NorA, NorB, NorC, MepA and MdeA [33]. Other multidrug efflux pumps expel only biocides, as is the case of QacA/B and Smr [33]. In general, specific efflux pumps can be found either in the chromosome or in plasmids, while multidrug efflux pumps are mainly located in the chromosome, with the exception of QacA/B and Smr, which have only been described in plasmids [33].

Despite the increasing number of *S. aureus* efflux pumps identified with the potential to contribute to the resistance towards clinically relevant antibiotics and other antimicrobial compounds, few studies have been undertaken to ascertain the collective and individual contribution of efflux systems to resistance phenotypes in clinical isolates [3, 5, 18], resulting in little information being available. One of the underlying reasons for this is the lack of established methods to assess efflux activity, mainly in the definition of threshold values for the attribution of "basal" *versus* "increased" levels for efflux activity, as well as the determination of the role of each pump on the overall efflux activity. Several approaches have been used to identify active efflux systems in bacteria, such as the use of radiolabelled substrates, fluorometric assays or the determination of the minimum inhibitory concentration (MIC) for different substrates in the presence of efflux inhibitors (EIs) [3, 16, 49]. In our group, we have developed methods based on ethidium bromide (EtBr), a substrate of the majority of the *S. aureus* multidrug efflux pumps, that has been proven reliable for the assessment of efflux activity in bacteria [44], namely the EtBr-agar cartwheel method [23], that allows the screening of large collections of clinical isolates to detect isolates with increased efflux activity. The monitoring of EtBr efflux in clinical isolates by real-time fluorometry [45] permits a more extensive characterization of that efflux activity and can be used to confirm, on a real-time basis, the results of the EtBr-agar cartwheel method. The use of EtBr has also been proven useful by other groups in detecting isolates with increased efflux activity [28]. The information obtained from these methods can then be

complemented by the determination of MICs of effluxable substrates in the presence of efflux inhibitors.

This work describes the use of these approaches to study the role played by efflux on the resistance to antimicrobial agents, including antibiotics and biocides, on a collection of *S. aureus* strains of clinical origin and how this efflux activity may contribute to the persistence of *S. aureus* cells overexpressing efflux pumps on the clinical environment.

6.2. Material and Methods

Bacterial strains and growth conditions. A collection of 52 ciprofloxacin-resistant *S. aureus* isolates was studied [3]. The pan-susceptible reference strain, *S. aureus* ATCC25923, and its ethidium bromide-adapted counterpart, ATCC25923$_{EtBr}$ [4] were used as controls. All strains were grown in tryptone soy broth (TSB) at 37°C with shaking or in tryptone soy agar (TSA) (Oxoid Ltd., Basingstoke, UK). The strain ATCC25923$_{EtBr}$ was grown in TSB/TSA supplemented with 50 mg/L of EtBr.

Antibiotics, biocides, dyes and efflux inhibitors. Antibiotics were purchased from different sources, as follows: ciprofloxacin (Fluka Chemie GmbH, Buchs, Switzerland) and norfloxacin (ICN Biomedicals Inc., Aurora, OH, USA). EtBr, biocides (benzalkonium chloride, tetraphenylphosphonium bromide, pentamidine isothionate salt, cetylpyridinium chloride, cetrimide, dequalinium chloride and chlorhexidine digluconate) and efflux inhibitors (thioridazine and verapamil) were acquired from Sigma-Aldrich (Madrid, Spain). All efflux inhibitors solutions were prepared in deionized water on the day of the experiment and kept protected from light.

EtBr-agar cartwheel method. For the EtBr-agar cartwheel method [23], each culture was swabbed onto TSA plates containing EtBr concentrations ranging from 0.5 to 2.5 mg/L in increments of 0.5 mg/L EtBr. *S. aureus* ATCC25923 and ATCC25923$_{EtBr}$ were used as negative and positive controls for efflux activity, respectively [4]. The plates were incubated at 37°C during 16 h, after which the minimum concentration of EtBr associated with the bacterial mass that produced fluorescence under UV light was recorded in a Gel-Doc XR apparatus (Bio-Rad, Hercules, CA, USA). Isolates showing

fluorescence at lower EtBr concentrations have potentially less active efflux systems than isolates for which fluorescence is only detected at higher concentrations of EtBr [23]. Thus, isolates were classified according to the emitted fluorescence registered, namely isolates showing emission of fluorescence at 0.5–1 mg/L EtBr were denominated EtBrCW-negative (with no potential active efflux systems); isolates showing emission of fluorescence at 1.5–2.0 mg/L EtBr were denominated EtBrCW-intermediate; and isolates emitting fluorescence only at the maximum concentration of EtBr tested (2.5 mg/L) were denominated EtBrCW-positive (with potential active efflux systems).

Efflux assays. Efflux assays by real-time fluorometry were performed in a Rotor-Gene 3000™ thermocycler, together with real-time analysis software (Corbett Research, Sydney, Australia) [45]. Cultures were grown in TSB medium at 37°C with shaking until an optical density at 600 nm (OD_{600}) of 0.6. The cells were collected by centrifugation at 13,000 rpm for 3 min and the pellet washed twice with a 1X phosphate buffered saline (PBS) solution. EtBr-loaded cells were prepared by incubating a cellular suspension with an OD_{600} of 0.3 with 0.25, 0.5 and 1 mg/L EtBr for EtBrCW-negative, -intermediate and -positive cultures, respectively, plus 200 mg/L of verapamil (a sub-inhibitory concentration) at 25°C for 60 min. After EtBr accumulation, cells were collected by centrifugation and resuspended in 1X PBS to an OD_{600} of 0.6. Several parallel assays were then run in 0.1 mL final volume corresponding to 0.05 mL of the EtBr loaded cells (final OD_{600} of 0.3) incubated with 0.05 mL of (1) PBS only, (2) glucose 0.8% only (final concentration of 0.4%), (3) 400 mg/L of verapamil only (final concentration of 200 mg/L) and (4) glucose 0.8% plus 400 mg/L of verapamil (final concentrations of 0.4% and 200 mg/L, respectively). Efflux assays were conducted in the Rotor-Gene 3000™ at 37°C, and the fluorescence of EtBr was measured (530/585 nm) at the end of every cycle of 10 sec, for a total period of 10 min. The raw data obtained was then normalized against data obtained from non-effluxing cells (cells from the control tube with only 200 mg/L VER), at each point, considering that these correspond to the maximum fluorescence values that can be obtained during the assay. The relative fluorescence thus corresponds to the ratio of fluorescence that remains per unit of time, relatively to the EtBr-loaded cells.

Minimum inhibitory concentration (MIC) determination. Cultures were grown in Mueller-Hinton broth (MH, Oxoid) at 37°C. MICs for antibiotics were determined by the two-fold broth microdilution method and evaluated according to the CLSI breakpoints [2]. MICs of EtBr and biocides were also determined using the two-fold broth microdilution method. After an 18 h incubation period at 37°C, the MIC values were recorded, corresponding to the lowest concentration of antimicrobial compound that presented no visible growth. To evaluate the effect of efflux inhibitors on the MIC values, parallel cultures were tested in media containing varying concentrations of the antimicrobial compound in the absence and presence of the efflux inhibitors thioridazine and verapamil at the sub-inhibitory concentrations of 12.5 mg/L and 200 mg/L, respectively, and equivalent bacterial inoculums. The cultures were incubated for 18 h and growth evaluated visually. All assays were determined in triplicate.

Screening of mutations conferring fluoroquinolone resistance. Internal fragments comprising the QRDR of *grlA* and *gyrA* genes were amplified using primers previously described [3]. The reaction mixture (0.05 mL) contained 2.5 U of Taq Polymerase (Fermentas Inc., Ontario, Canada), 1X Taq buffer (Fermentas), 25 pmol of each primer, 0.2 mM of dNTP and 1.75 mM of $MgCl_2$. The PCR reactions were conducted in a thermocycler Mastercycler personal 5332 (Eppendorf AG, Hamburg, Germany). The amplification conditions were as follows: DNA was denatured at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 50 °C for 30 sec and extension at 72 °C for 1 min, followed by a step of final extension at 72 °C for 5 min. Amplification products were purified and sequenced in both strands using the same set of primers. Sequences were analyzed and aligned using the freeware programs, BioEdit and ClustalW, respectively.

6.3. Results and discussion

6.3.1. Evaluation of efflux activity

The EtBr-agar cartwheel (EtBrCW) method is a practical methodology to assess the presence of increased efflux activity in large collections of clinical isolates of

different bacterial species [23]. This method allows the comparison of different isolates on the basis of their capacity to extrude EtBr. The isolates are streaked in solid media containing increasing concentrations of EtBr and the fluorescence emitted, which is inversely proportional to their capacity to extrude the compound, is compared to the fluorescence of control strains. Using this approach to test a collection of 52 ciprofloxacin-resistant *S. aureus*, we could discriminate these isolates in three distinct groups: a group of twelve isolates that showed fluorescence only at the highest EtBr concentration tested, presumably with increased efflux activity and designated EtBrCW-positive; a group of thirty-three isolates that showed fluorescence at the lowest EtBr concentrations tested and denominated EtBrCW-negative; and a third group of seven isolates showing fluorescence at intermediate concentrations of EtBr and denominated EtBrCW-intermediate isolates [3] (Figure 6.1).

Further characterization of these isolates by a fluorometric assay that detects EtBr efflux by assessing in real-time the loss of EtBr fluorescence in bacterial cells previously loaded with this dye enabled us to corroborate the preliminary characterization of the isolates by the EtBrCW method (Figure 6.1). In particular, the increased efflux activity present in EtBrCW-positive isolates (Figure 6.1, red) is demonstrated by the lack of fluorescence in EtBr-agar plates together with a prompt EtBr efflux by real-time fluorometry, whereas EtBrCW-negative isolates (Figure 6.1, blue) emit a strong fluorescence in EtBr-agar plates and show only slight EtBr efflux. On the other hand, EtBrCW-intermediate isolates show intermediate fluorescence and EtBr efflux (Figure 6.1, orange). This analysis also showed that basal efflux activity is always present in *S. aureus*, as shown by the reference strain. ATCC25923 (Figure 6.1, green).

Altogether, these two EtBr-based assays proved to be valuable tools to screen for increased efflux activity in clinical isolates of *S. aureus*, making it possible to differentiate strains with varying levels of efflux activity. As stated previously, EtBr is a common substrate of multidrug efflux pumps, which can also extrude other antimicrobial compounds, such as the antibiotics fluoroquinolones and biocides and, thus, used as a screening marker for efflux, leading to resistance towards fluoroquinolones and biocides.

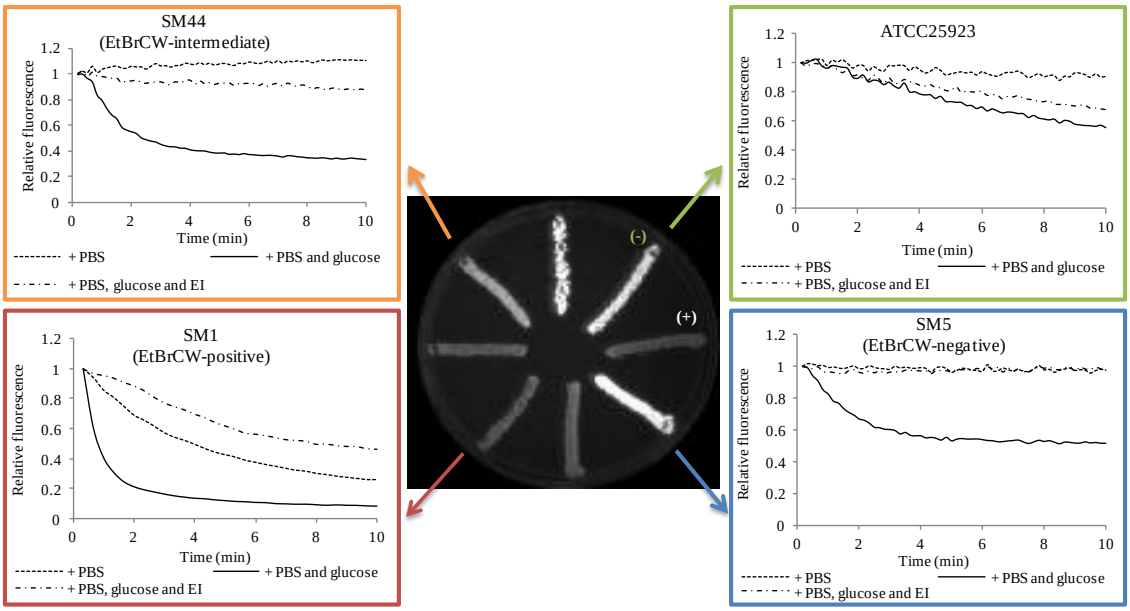


Figure 6.1. Characterization of reference and clinical isolates according to their efflux capacity. In green: reference strain *S. aureus* ATCC25923; in blue: EtBr-agar cartwheel (EtBrCW)-negative isolate SM5; in orange: EtBrCW-intermediate isolate SM44; in red: EtBrCW-positive isolate SM1. Central figure: screening of efflux activity by the EtBrCW method. Tryptone soy agar (TSA) plate supplemented with 2.5 mg/L EtBr streaked with representative isolates. (−) and (+): strains ATCC25923 and ATCC25923$_{EtBr}$, used respectively as negative and positive controls for EtBr efflux. Graphics: evaluation of efflux activity by real-time fluorometry. Efflux assays for representative isolates are shown for cells resuspended in Phosphate buffered saline (PBS), in PBS plus glucose (0.4%) or PBS plus glucose and the efflux inhibitor verapamil (at the sub-inhibitory concentration of 200 mg/L). The data presented was normalized against the data obtained in conditions of no efflux (absence of glucose and presence of 200 mg/L of verapamil).

6.3.2. Contribution of efflux to fluoroquinolone resistance

Fluoroquinolones are a class of antibiotics that possess a broad spectra of activity, including methicillin-resistant *S. aureus* (MRSA) [48]. However, the swift development of resistance to these antibiotics has impaired their clinical relevance [12]. At present, in Europe, around 25% of *S. aureus* isolates are resistant to fluoroquinolones, a percentage that increases to 90% among MRSA isolates [6].

Resistance to fluoroquinolones in *S. aureus* is usually associated with the occurrence of mutations in the target genes, *grlA/B* and *gyrA/B*, that code for Topoisomerase IV (GrlA/B) and DNA gyrase (GyrA/B) proteins, respectively [12]. These mutations usually occur in a precise region denominated quinolone resistance-determining region (QRDR) and generate proteins with lower affinity for fluoroquinolones [12]. Several studies have shown that Topoisomerase IV is the

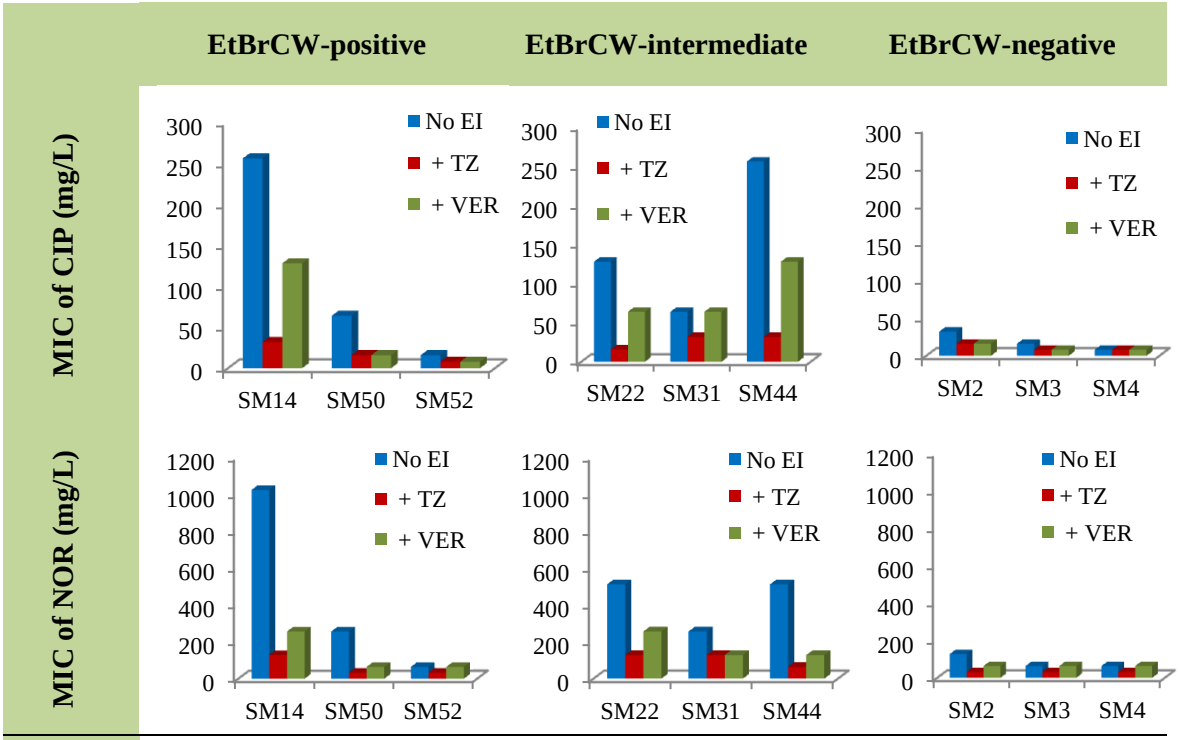
primary target of fluoroquinolones in *S. aureus*. Accordingly, *in vitro* studies have demonstrated that emergence of fluoroquinolone resistance is associated with acquisition of mutations first in the *grlA* gene followed by mutations in the *gyrA* gene [7]. Also, fluoroquinolone resistant clinical isolates with mutations only in the *gyrA* gene are uncommon [40]. Moreover, quinolone resistant isolates with a single or double mutation in the *grlA* gene present high-level resistance when a *gyrA* mutation is acquired [27, 40]. The occurrence of mutations in the *grlB* and *gyrB* genes has been shown to be infrequent [12]. Altogether, mutations in the QRDR of these genes are linked to high-level resistance to fluoroquinolones in *S. aureus* clinical isolates [12].

Resistance to fluoroquinolones mediated by efflux has been described in *S. aureus* clinical isolates for the last two decades [7, 11, 13, 17, 26, 34-36, 41, 49]. However, these studies related only to the role of the NorA efflux pump. Nowadays, it is known that at least three other multidrug efflux pumps, namely NorB, NorC and MepA, have been described as having fluoroquinolones as a substrate [15, 42, 43], although their actual contribution to clinical fluoroquinolone resistance remains uncertain. A few studies have been conducted with clinical isolates where an association between fluoroquinolone resistance and these efflux systems was explored [3, 5, 18].

The effect of the known efflux inhibitors, thioridazine (TZ) and verapamil (VER) [3], on the MIC levels of fluoroquinolones was evaluated, and mutations conferring fluoroquinolone resistance were screened. Reserpine is usually used to assess efflux activity in *S. aureus*, but it was not tested in this study, since previous data by our group has revealed that this compound has a mild inhibitory activity [3]. The isolates presented in Figure 6.2 are representative of each of the groups established previously, according to efflux capacity. Detailed data on MIC values is provided in Supplementary Table S1 (see annex A).

The data presented in Figure 6.2 shows that independently of the mutations carried by each strain in both *grlA* and *gyrA* genes, which alter the target affinity of these antibiotics, efflux is an important component of the resistance level. This can be observed in the effect of the efflux inhibitor, TZ, on the MICs of the two fluoroquinolones tested for these isolates. The inhibitory effect of TZ was shown to be higher for strains with higher efflux activity, namely the ones classified as EtBrCW-positive or EtBrCW-intermediate, for which a two- to eight-fold reduction in the MICs

of the two fluoroquinolones was observed, whereas this reduction was only two-fold for the EtBrCW-negative isolates. Verapamil showed a weaker effect, resulting in MIC reductions that ranged from none to four-fold for EtBrCW-positive and -intermediate isolates and none to two-fold for EtBrCW-negative isolates. Comparing the data gathered for the EtBrCW-intermediate isolates with the data of the remaining isolates, it could be observed that EtBrCW-intermediate isolates are more similar to EtBrCW-positive isolates. All these isolates carried mutations in both *grlA* and *gyrA* genes that have been described in the literature as being involved in high-level resistance to fluoroquinolones [35, 46]. Accordingly, our isolates present ciprofloxacin MICs that range between 16 to 256 mg/L for EtBrCW-positive and -intermediate isolates and 8 to 32 mg/L for EtBrCW-negative isolates; whereas the norfloxacin MICs vary between 64 to 1,024 mg/L for EtBrCW-positive and -intermediate isolates, and 64 to 128 mg/L for EtBrCW-negative isolates. However, part of this resistance can be attributable to efflux, as seen in the degree of MIC reductions by the efflux inhibitors tested in Figure 6.2. We also observed that although isolates carrying a double mutation in *grlA* and a single mutation in *gyrA* presented higher MICs, as described in the literature, they also suffered higher MIC reductions with the efflux inhibitors, particularly with TZ (two- to eight-fold) in comparison with isolates carrying a single mutation in both genes (none to two-fold). Regardless of the type of combination of QRDR mutations presented by the isolates, the presence of TZ reduced the MICs of ciprofloxacin to 8–32 mg/L and the MICs of norfloxacin to 32–128 mg/L (Figure 6.2 and Supplementary Table S1 – see annex A). However, the fluoroquinolone resistance phenotype was not fully reverted by TZ, since all isolates remained resistant to either ciprofloxacin or norfloxacin in the presence of the inhibitor, as expected, due to the presence of mutations.



QRDR mutations	SM14	SM50	SM52	SM22	SM31	SM44	SM2	SM3	SM4
GrlA	S80Y E84G	S80F E84K	S80Y	S80Y E84G	S80F	S80Y E84G	S80F E84K	S80F E84G	S80F
GyrA	S84L	S84L	S84L	S84L	S84L	S84L	S84L	S84L	S84L

Figure 6.2. Effect of the efflux inhibitors (EIs) thioridazine (TZ) and verapamil (VER), at subinhibitory concentrations (12.5 mg/L and 200 mg/L, respectively), on the MIC values of ciprofloxacin (CIP) and norfloxacin (NOR) for representative isolates of the EtBrCW-positive, EtBrCW-intermediate and EtBrCW-negative groups, each carrying different mutations conferring fluoroquinolone resistance (data for EtBrCW-positive and -negative strains from our previous study, [3]).

These results reveal that efflux is an important contributor to fluoroquinolone resistance in *S. aureus.* While mutations in the QRDR of *grlA* and *gyrA* genes confer resistance up to a certain level, in particular 8 to 32 mg/L for ciprofloxacin and 32 to 128 mg/L for norfloxacin, the remaining resistance may be attributable to efflux, thus demonstrating that efflux is a relevant component of the level of fluoroquinolone resistance in these clinical isolates.

This last observation is further supported by data on strain SM15, classified as EtBrCW-intermediate and showing low level resistance to fluoroquinolones and the

single isolate among the EtBrCW-intermediate isolates that carry solely a single mutation in *grlA* QRDR and no mutation in *gyrA* (Figure 6.3). This strain presented MICs of 8 mg/L for ciprofloxacin and 16 mg/L for norfloxacin, which are near the breakpoint concentrations for an isolate to be considered resistant to these antibiotics (according to CLSI guidelines [2]). These values are in accordance to data in the literature for isolates carrying a single GrlA mutation [46]. However, the addition of efflux inhibitors lead to a reduction of these MIC levels, which, in the particular case of TZ, drop to the susceptibility levels for both ciprofloxacin and norfloxacin (1 mg/L and 4 mg/L, respectively) (Figure 6.3).

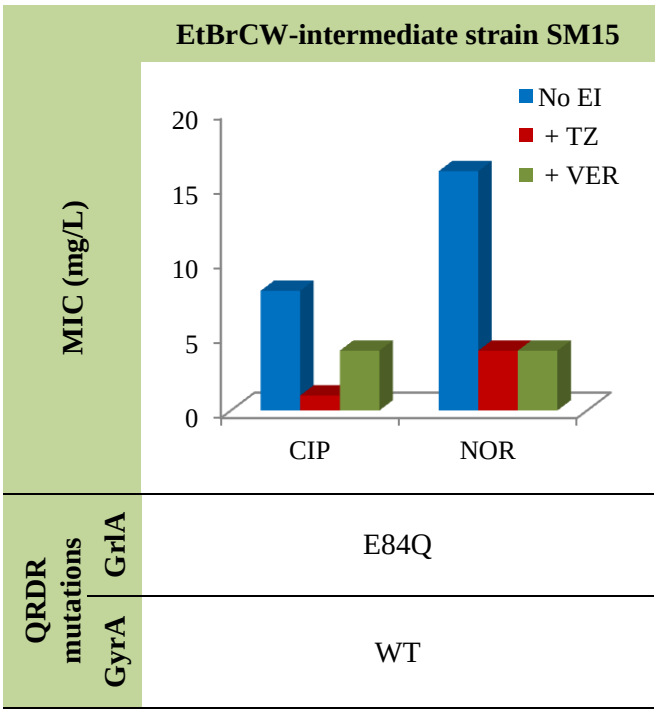


Figure 6.3. Effect of the efflux inhibitors (EIs) thioridazine (TZ) and verapamil (VER), at a subinhibitory concentration (12.5 mg/L and 200 mg/L, respectively), on the MIC values of ciprofloxacin (CIP) and norfloxacin (NOR) for the EtBrCW-intermediate strain SM15. WT: wild-type sequence (no mutations).

These results strengthen further the importance of efflux on fluoroquinolone resistance in *S. aureus*, in particular in the early stage of the acquisition of mutations in the QRDR of *grlA* and *gyrA* genes. Strain SM15 may represent an intermediate stage of the emergence of fluoroquinolone resistance, with balanced contributions from both efflux and mutation to the resistance phenotype.

In fact, recent studies from independent groups, working with different bacterial species, suggest that efflux systems may be a first response of the cell to cope with antimicrobial agents, enabling the cell to survive and acquire other, more stable resistance mechanisms, such as target gene mutations for fluoroquinolones, that will then provide a high-level resistance phenotype, as was recently demonstrated for *Escherichia coli* [38] and *Mycobacterium tuberculosis* [20]. In *S. aureus*, some evidence has also been found for this role of efflux pumps as a first-line defense mechanism towards noxious compounds [22, 39] that are supported by data on clinical strains [3, 5, 14, 18].

6.3.3. Contribution of efflux to biocide reduced susceptibility

Biocides differ greatly from antibiotics in their mechanism of action; whereas antibiotics have precise cell targets, biocides usually act upon several cellular targets [25]. They have an important role in infection control in healthcare settings, where they are currently used in a variety of products that are applied in the washing and disinfection of the environment and medical devices. They are also used as antiseptics for patients and healthcare professionals in hand hygiene, skin disinfection prior to invasive procedures and mucous disinfection [25]. Among these biocides, antiseptic formulations containing chlorhexidine or quaternary ammonium compounds, together with alcohol-based preparations, are the most commonly used for skin disinfection and hand hygiene, respectively [9]. Apart from healthcare settings, these compounds are widespread in industry, being also increasingly employed in the community setting [8]. Concern regarding the emergence of clinical strains showing reduced susceptibility or tolerance to biocides has been increasing in the last decade, with particular focus on the potential role of biocides as a selective force of antibiotic-resistant bacteria [21].

Reduced susceptibility to biocides in *S. aureus* is mainly associated with efflux pumps that are encoded in plasmids, including the efflux systems, QacA/B and Smr [33]. Nevertheless, the several chromosomally-encoded multidrug efflux pumps that have been described so far in *S. aureus* also have in their substrate profile a wide variety of biocides. Therefore, it is important to ascertain the contribution of these efflux systems to the biocide "susceptibility" profile in clinical isolates.

The collection of 52 ciprofloxacin-resistant *S. aureus* isolates was also tested for susceptibility to biocides by determination of MICs of several biocides, namely the quaternary ammonium compounds, cetrimide, cetylpyridinium chloride and benzalkonium chloride, the bisbiguanidine chlorhexidine digluconate, pentamidine, tetraphenylphosphonium bromide and dequalinium chloride. MICs of EtBr were also determined, since this compound was used as a marker for efflux activity. Data for representative isolates can be found in Figure 6.4. Among the compounds tested, it could be observed that the MICs for EtBrCW-positive and -intermediate isolates were generally higher than the ones for EtBrCW-negative isolates. In particular, EtBrCW-positive and -intermediate isolates presented MICs in the following range: EtBr, 8 to 16 mg/L; the quaternary ammonium compounds cetrimide, 4 to 8 mg/L, cetylpyridinium chloride, 1 to 4 mg/L and benzalkonium chloride, 2 to 4 mg/L; tetraphenylphosphonium bromide, 16 to 64 mg/L; chlorhexidine digluconate, 0.00006% to 0.000125%, and dequalinium chloride, 4 to 16 mg/L. The range of MICs for the EtBrCW-negative isolates varied as follows: EtBr, 2 to 8 mg/L; cetrimide, 2 mg/L; cetylpyridinium chloride, 0.5 mg/L; benzalkonium chloride, 1 mg/L, tetraphenylphosphonium bromide, 16 to 32 mg/L, chlorhexidine digluconate, 0.00003% to 0.00006%; and dequalinium chloride, 2 to 4 mg/L (detailed MIC data is provided in Supplementary Tables S2 and S3). No significant difference was found between the groups of isolates for the MICs of pentamidine (data not shown). In sum, the EtBrCW-positive and -intermediate isolates presented MIC values that were two- to eight-fold higher than the ones presented by the EtBrCW-negative isolates (Figure 6.4). Although this difference is not extensive, it reveals that the efflux-positive and -intermediate isolates can withstand higher concentrations of these biocides. Moreover, it demonstrated a variation of the MICs of biocides according to the efflux capacity of the three groups of isolates (Figure 6.4).

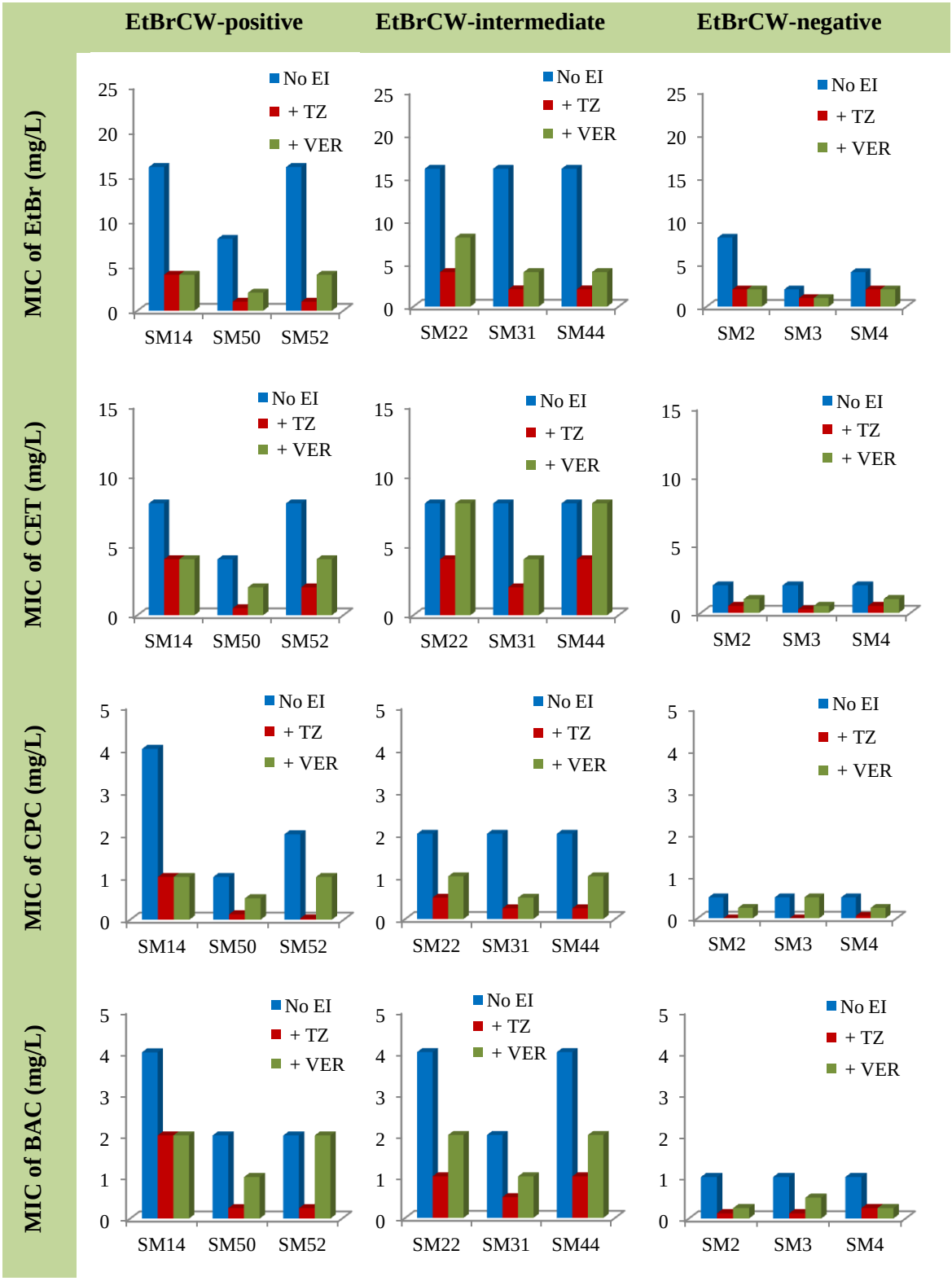


Figure 6.4. Effect of the efflux inhibitors (EIs) thioridazine (TZ) and verapamil (VER), at subinhibitory concentrations (12.5 mg/L and 200 mg/L, respectively), on the MIC values of several biocides for representative isolates of the EtBrCW-positive, EtBrCW-intermediate and EtBrCW-negative groups. EtBr: ethidium bromide; CET: cetrimide; CPC: cetylpyridinium chloride; BAC: benzalkonium chloride.

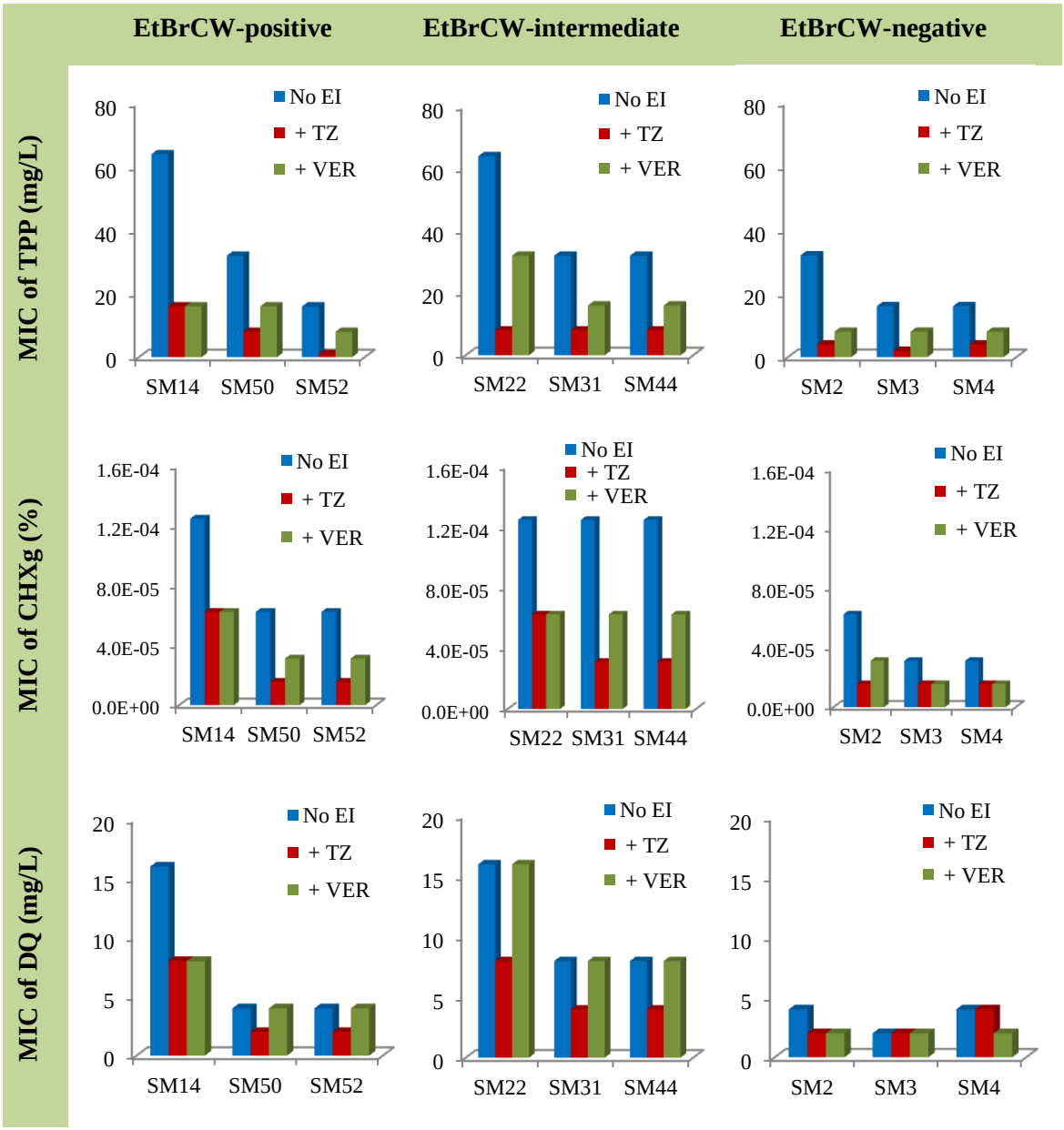


Figure 6.4. (*Cont.*) Effect of the efflux inhibitors (EIs) thioridazine (TZ) and verapamil (VER), at subinhibitory concentrations (12.5 mg/L and 200 mg/L, respectively), on the MIC values of several biocides for representative isolates of the EtBrCW-positive, EtBrCW-intermediate and EtBrCW-negative groups. TPP: tetraphenylphosphonium bromide; CHXg: chlorhexidine digluconate; DQ: dequalinium chloride.

To assure that the difference observed in the MIC values of the biocides and EtBr was the result of the higher efflux capacity of the EtBrCW-positive and EtBrCW-intermediate isolates, the effect of TZ and VER on the MICs of these compounds was also evaluated (Figure 6.4 and Supplementary Tables S2 and S3 – see annex A).

The effect of the efflux inhibitors upon the MIC values of the biocides selected showed that efflux activity has a strong involvement in the reduced susceptibility of *S. aureus* to biocides. For all isolates tested, independently of their efflux capacity, the MICs of all the biocides were reduced two- to 16-fold in the presence of TZ and none to eight-fold (mostly, about two-fold reduction) in the presence of VER, with the highest inhibitory effects observed for the quaternary ammonium compounds and for tetraphenylphosphonium bromide. The biocide for which this effect was lower was dequalinium chloride, with none to two-fold reduction of MICs in the presence of the efflux inhibitors. It could also be observed, in general, that for EtBrCW-positive and -intermediate isolates, the efflux inhibitors could reduce the MICs of the several compounds to levels similar or lower than the ones presented by the EtBrCW-negative isolates. Altogether, these results indicate that efflux activity contributes to reduced susceptibility to biocides in *S. aureus*.

We have previously referred to the lower capacity of fluoroquinolone MIC reduction by VER, when compared to TZ [3]. Here, we provide experimental data on this difference and show that the same effect is observable for biocides, as well.

Among the EtBrCW-positive isolates, SM52 is the only to carry a plasmid with the gene for the efflux pump Smr [3]. This pump is associated with low-level resistance to biocides and EtBr [10] and is found in a low prevalence (around 10%) in clinical *S. aureus* isolates [19, 24, 50]. The MIC values presented by the isolate SM52 are equal or lower than the ones of the remaining EtBrCW-positive and -intermediate isolates (that carry no Smr or QacA/B efflux pump), thus showing that the potentially active chromosomal multidrug efflux pumps can confer a resistance level to biocides similar or higher than the Smr plasmid-encoded efflux pump.

The results described suggest that active efflux systems can be responsible for reduced susceptibility to biocides in *S. aureus*. The concentration in-use in which these compounds are applied for washing and disinfection in healthcare settings is higher than the MICs values determined for these isolates. For example, chlorhexidine is used in concentrations that range between 0.2 to 4%, much higher than the highest MIC found in these isolates. This may suggest that this observed reduced susceptibility to biocides is not clinically relevant. Nevertheless, the misuse of these biocide formulations, especially in terms of the application time, together with contaminating residues left

after use, may provide opportunities for more tolerant bacteria to be maintained and proliferate in these environments [1, 37, 47]. Furthermore, concern arises regarding biocide-resistant/tolerant strains and their role in the selection of antibiotic-resistant strains. Studies have related contradictory data concerning the relation between antibiotic and biocide resistance, but much evidence has been gathered that supports the potential co-selection of strains with reduced susceptibility to biocides by antibiotic-resistant bacteria, and *vice versa*. In the particular case of *S. aureus*, studies have shown that exposure to biocides can induce the overexpression of multidrug efflux pumps in both reference and clinical strains, leading to reduced susceptibility to the inducing biocide, as well as to other biocides and to antibiotics [4, 14]. These findings strengthen the importance of increasing our knowledge of efflux as a resistance mechanism in *S. aureus*.

6.4. Conclusions

The results described demonstrate that the two EtBr-based approaches used, namely the EtBrCW method and real-time fluorometry, are valuable techniques to screen and characterize efflux activity in clinical isolates of *S. aureus*. These isolates were classified according to their capacity to efflux EtBr and the MIC determination in the presence of efflux inhibitors allowed the correlation of this efflux activity with resistance to fluoroquinolones and some biocides, including quaternary ammonium compounds and chlorhexidine, antimicrobials widely used in healthcare settings. These results show that EtBr is indeed a good screening marker for efflux activity leading to resistance to fluoroquinolones and biocides.

The data compiled in this study, together with results from previous studies from our group and other colleagues, indicate that efflux is a major mechanism in the first stage of development of resistance to antimicrobial compounds, in this case, fluoroquinolones. That is, activation of efflux systems by fluoroquinolones could promote *S. aureus* survival in “stress conditions”, allowing the bacteria to acquire and accumulate target gene mutations that are associated with high-level resistance. Furthermore, the demonstration that this same efflux activity can sustain higher tolerance of *S. aureus* cells to clinically relevant antiseptics and disinfectants, which

could also, in turn, potentiate antibiotic resistance, strengthen the importance of this long neglected resistance mechanism to the persistence and proliferation of antibiotic/biocide-resistant *S. aureus* in the hospital environment.

6.5 References

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CHAPTER 7

Efflux as a first-line response to antimicrobial agents in *S. aureus*

7. Efflux as a first-line response to antimicrobial agents in *S. aureus*

Summary

In *S. aureus*, efflux-mediated resistance to antimicrobial agents, in particular fluoroquinolones, has been considered clinically non-relevant and consequently neglected in comparison with mutational resistance. The balance between efflux and mutation during the development of resistance to fluoroquinolones and other antimicrobials is the focus of this study. Two ciprofloxacin-resistant *S. aureus* clinical strains, differing in their efflux capacity, and the pan-susceptible reference strain ATCC25923 were subjected to exposure to constant concentrations of efflux inducers, namely ciprofloxacin, ethidium bromide and cetrimide and the cell response to these agents was monitored. Susceptibility testing by determination of MICs for the exposed strains revealed that exposure to those agents resulted in strains with reduced susceptibility to fluoroquinolones, biocides and dyes, independently of the inducer or its concentration. The emergence of the multidrug resistance phenotypes was correlated with an increase of efflux capacity for the exposed strains by determination of MICs in the presence of efflux inhibitors together with fluorometric detection of EtBr efflux. Gene expression assays of the strains exposed to efflux inducers disclosed a temporal pattern for expression of multidrug efflux pump genes consisting in an early-response with high levels of expression of several efflux pump genes followed by a late-response, characterized by overexpression of specific genes. The overall cell response to the efflux inducers varied according to the strains original status of the efflux activity, being more pronounced for the strains with an initial basal efflux activity. In the particular case of the susceptible strain ATCC25923, exposure to ciprofloxacin allowed us to witness the step-wise development of resistance to fluoroquinolones, with a first efflux-mediated response, followed by the occurrence of one mutation in the *grlA* target gene that resulted in phenotypic resistance to fluoroquinolones.

7.1 Introduction

S. aureus is a frequent human colonizer as well as one of the most important pathogenic bacteria causing mild to life-threatening infections. Adding to the potential pathogenicity and virulence, acquisition and/or development of resistance to antibiotics

and other antimicrobial compounds is of foremost importance in *S. aureus* as the occurrence of strains with a phenotype of resistance to multiple antimicrobial compounds (MDR phenotype) is common. In particular, methicillin-resistant *S. aureus* (MRSA) strains, which are resistant to all β-lactam antibiotics and potentially to other antimicrobial compounds, have become a major problem in healthcare settings and in the community [1]. These MRSA strains pose a major health concern as they are associated with higher mortality rates than methicillin-susceptible *S. aureus* (MSSA) strains [33].

Fluoroquinolones are a class of antibiotics that target the *S. aureus* topoisomerase IV (GrlA/B) and DNA gyrase (GyrA/B) inhibiting the DNA replication process [14]. Resistance to fluoroquinolones emerges swiftly and has been mainly attributed to the occurrence of spontaneous mutations in the quinolone resistance-determining region (QRDR) of the target genes *grlA*/*B* and *gyrA*/*B* [14]. In Europe, around 25% of *S. aureus* invasive clinical isolates is resistant to fluoroquinolones, a rate that increases to 90% among MRSA isolates [10]. Although fluoroquinolones are not usually applied in the treatment of staphylococcal infections, they are one of the most used classes of antibiotics in hospitals [11]. The intensive presence of these antibiotics in hospitals has been pointed out as a main selective factor for the emergence and dissemination of fluoroquinolone resistance in *S. aureus* [15]. In addition, evidence suggests that fluoroquinolone resistance may act as a selective advantage for MRSA strains in comparison with MSSA strains [15].

Resistance to fluoroquinolones may also arise by the extrusion of the antibiotic by multidrug efflux pumps [27]. Efflux-mediated resistance has been reported in *S. aureus* clinical isolates in the last two decades [23, 24, 29], although its contribution to the resistance levels registered has been considered low and thus clinically non-relevant [13]. Several genes encoding multidrug efflux pumps have been identified in the *S. aureus* chromosome and their products characterized, including NorA, NorB, NorC, MepA and MdeA [7]. All these chromosomally-encoded efflux systems are able to extrude fluoroquinolones from the cell. The few studies conducted to ascertain their contribution to fluoroquinolone resistance have reported a correlation between their activity and a reduced susceptibility to these antibiotics [5, 9, 18, 19]. Moreover, this same efflux activity could be linked to decreased susceptibility to additional

antimicrobial compounds such as biocides and dyes [5, 6, 9], thus highlighting the potential of these efflux pumps to convey a MDR phenotype to *S. aureus* strains.

Recent studies on different bacteria provided additional data supporting the premise that efflux plays an important role in the emergence of resistance to antimicrobial agents. Indeed, data from both *Escherichia coli* and *Mycobacterium tuberculosis* demonstrate that efflux may be the cells first response to cope with these compounds, allowing the cell to endure their presence and noxious effects until acquisition of a more stable resistance mechanism, such as mutations, that will then provide a high-level resistance phenotype [21, 30]. A few studies on *S. aureus*, also evidenced the role of efflux as a first-line defence mechanism towards noxious compounds [22, 31], a hypothesis that has been supported by data with clinical isolates [5, 9, 16].

In sum, evidence has been gathered in literature sustaining efflux as a major player in the development of resistance towards different antimicrobial agents in *S. aureus*. In this study, we aimed to enlighten the relation between efflux and mutation throughout the process of emergence of resistance in *S. aureus*, by exposing three strains to sub-inhibitory or inhibitory concentrations of distinct antimicrobial agents that are known substrates of multidrug efflux pumps, namely the fluoroquinolone ciprofloxacin (CIP), the biocide cetrimide (CET) and the dye ethidium bromide (EtBr). The expression of genes coding for the main multidrug efflux pumps and their regulators was assessed at different time points of the exposure process and correlated with the resistance level towards fluoroquinolones and other antimicrobial agents, and the temporal acquisition of mutations during the time-course of the exposure process.

7.2 Material and Methods

Bacterial strains. A brief summary on the main characteristics of the three *S. aureus* strains studied in this work is given in Table 7.1. *S. aureus* ATCC25923 is a fully susceptible reference strain, used as control in susceptibility testing, SM50 and SM2, are two MRSA clinical strains, selected among a collection of 52 ciprofloxacin-resistant *S. aureus* isolates [5, 6]. SM50 and SM2 are genetically related, corresponding to two sub-types of the same PFGE type that differ in one SmaI-fragment of ca. 400 kb. They

carry the same set of QRDR mutations in the *grlA* and *gyrA* genes but show a different level of resistance to fluoroquinolones, with strain SM50 being more resistant than SM2. This higher resistance of SM50 has been previously correlated with an increased efflux activity, which was also associated with increased resistance to biocides and EtBr. In comparison, strain SM2 presents a basal efflux activity. Strain ATCC25923 also shows a residual efflux activity that has been considered the level of basal efflux activity for *S. aureus* [5, 6].

Table 7.1. Characterization of the *S. aureus* strains used in this study.

Strains	Description	Reference
ATCC25923	Pan-susceptible reference strain QRDR mutation: GrlA – P144S Presents basal efflux activity	[5, 6]
SM2	Clinical isolate, methicillin-resistant, ciprofloxacin-resistant PFGE-type B2; QRDR mutations: GrlA – S80Y/E84K, GyrA – S84L Presents basal efflux activity	
SM50	Clinical isolate, methicillin-resistant, ciprofloxacin-resistant PFGE-type B1; QRDR mutations: GrlA – S80Y/E84K, GyrA – S84L Presents increased efflux activity	

QRDR: quinolone resistance-determining region; P: proline; S: serine; Y: tyrosine; E: glutamic acid; K: lysine; L: leucine: PFGE: pulsed-field gel electrophoresis.

Antimicrobial agents and efflux inhibitors. The compounds used in this study were acquired from different sources, as follows: CIP, levofloxacin, chlorhexidine diacetate (Fluka Chemie GmbH, Buchs, Switzerland); norfloxacin (ICN Biomedicals Inc., Aurora, OH, USA); oxacillin, penicillin, vancomycin, chloramphenicol, tetracycline, gentamicin, EtBr, CET, pentamidine isothionate salt, cetylpyridinium chloride, benzalkonium chloride, tetraphenylphosphonium bromide, chlorhexidine digluconate, dequalinium chloride, verapamil and thioridazine (Sigma-Aldrich, St. Louis, MO, USA). Stock solutions of the efflux inhibitors verapamil and thioridazine were prepared in deionized water on the day of the experiment and kept protected from the light.

Exposure procedure. The *S. aureus* strains ATCC25923, SM50 and SM2 were serially exposed during 20 days to constant concentrations equivalent to ½ the minimum inhibitory concentration (MIC) or the MIC itself, for each strain, of the three efflux

inducers used in this study: EtBr, CIP or CET. Cultures were grown overnight in tryptone soya broth (TSB, Oxoid Ltd., Basingstoke, UK) and then diluted 100-fold in TSB (control) or in TSB supplemented with the efflux inducers, as follows: EtBr at ½ the MIC, 3 mg/L for ATCC25923, 4 mg/L for SM50 and SM2; EtBr at the MIC, 6.25 mg/L for ATCC25923 and 8 mg/L for SM50 and SM2; CIP at ½ the MIC, 0.125 mg/L for ATCC25923, 16 mg/L for SM2 and 32 mg/L for SM50, CIP at the MIC, 0.25 mg/L for ATCC25923, 32 mg/L for SM2 and 64 mg/L for SM50; CET at ½ the MIC, 1 mg/L for ATCC25923 and SM2, 2 mg/L for SM50; CET at the MIC, 2 mg/L for ATCC25923 and SM2, 4 mg/L for SM50. The cultures were incubated at 37 ºC with shaking and after 18 h an aliquot was diluted 100-fold in media supplemented with the same concentration of the inducer and grown in the same conditions. This procedure was repeated through 20 passages in 20 days, after the first culture (P1) was obtained (Figure 7.1). The response of the strains to the efflux inducers was monitored in several time points. Susceptibility towards the inducers was tested in the first three days and in the 20^{th} day of exposure. Susceptibility to a wide panel of antibiotics, biocides and dyes and assessment of EtBr efflux activity were also monitored at the beginning (day 0) and at the end of the exposure procedure (day 20). Analysis of the expression of genes coding for the main multidrug efflux pumps and their regulators was performed during day 1 of exposure at mid exponential growth phase (optical density at 600 nm, OD_{600} of 0.6) and at the 18^{th} h of growth, as well as at the 18^{th} h of growth of the 20^{th} day of exposure. At these time points, an aliquot of 2 mL of culture was transferred to a mixture of 1:1 acetone:ethanol and immediately kept at -20ºC for total RNA extraction. For all strains and all growth conditions, a stock was made of each passage in 10% (v/v) glycerol and kept at -80ºC.

Macrorestriction analysis. Cultures were typed by pulsed-field gel electrophoresis (PFGE) analysis, using well-established protocols. Briefly, agarose disks containing intact chromosomal DNA were prepared as previously described [3] and restricted with SmaI, according to the manufacturer's recommendations (New England Biolabs, Ipswich, MA, USA). Restriction fragments were then resolved by PFGE, which was carried out in a contour-clamped homogeneous electric field (CHEF) apparatus (CHEF-DRII, Bio-Rad, Hercules, CA, USA) as previously described [3]. Lambda ladder PFG marker (New England Biolabs) was used as a molecular weight marker.

Determination of MICs. Cultures were grown in Mueller-Hinton broth (MH, Oxoid) at 37°C. MICs of antibiotics were determined by the two-fold broth microdilution method and evaluated according to CLSI breakpoints [4]. MICs of biocides, dyes and efflux inhibitors were also determined using the two-fold broth microdilution method. After an 18 h incubation period at 37°C, the MIC values were recorded, corresponding to the lowest concentration of antimicrobial compound that presented no visible growth. **MICs in the presence of efflux inhibitors.** The inhibitory effect of the efflux inhibitors on the susceptibility towards the different antimicrobials tested was evaluated using the two-fold broth microdilution method in medium containing varying concentrations of the antimicrobial compound, a sub-inhibitory concentration of the efflux inhibitor and a bacterial inoculum corresponding to the one used for MIC determination. The cultures were incubated for 18 h and growth evaluated visually. The final concentrations of the efflux inhibitors used corresponded to half, or below, the MICs determined for each efflux inhibitor, as follows: thioridazine (TZ) (12.5 mg/L) and verapamil (VER) (200 mg/L), to guarantee that the inhibitors did not affect cell viability. All assays were performed in triplicate.

Assessment of EtBr efflux activity. This method allows the real-time fluorometric detection of the efflux of EtBr accumulated inside cells using a Rotor-Gene 3000™ thermocycler together with real-time analysis software (Corbett Research, Sydney, Australia) [34]. For efflux assays, cultures were grown in TSB medium at 37°C with shaking until mid exponential growth phase (OD_{600} of 0.6) was reached. At this point, cells were collected by centrifugation at 13,000 rpm for 3 min and the pellet washed twice with a 1X Phosphate Buffered Saline (PBS) solution. The OD_{600} of the cellular suspension was then adjusted to 0.3 in 1X PBS. EtBr-loaded cells were prepared by incubating the cellular suspension with the most suitable EtBr concentration for each culture (0.125 mg/L for ATCC25923_P0, ATCC25923_CIP(½MIC)_P20, ATCC25923_CIP(MIC)_P20, ATCC25923_CET(½MIC)_ P20, SM2_P0, SM2_CET(½MIC)_P20 and SM50_CET(½MIC)_P20; 0.25 mg/L for ATCC25923_EtBr(½MIC)_P20, ATCC25923_CET(MIC)_P20, SM2_CET(½MIC)_P20, SM2_CET(MIC)_P20, SM50_ P0, SM50_EtBr(½MIC)_P20, SM50_EtBr(MIC)_P20, SM50_CIP(MIC)_P20: 0.5 mg/L for ATCC25923_EtBr(MIC)_P20, SM2_EtBr(½MIC)_P20, SM2_EtBr(MIC)_P20,

SM2_CIP(MIC)_P20, SM50_CIP(½MIC)_P20) and 200 mg/L of verapamil, the efflux inhibitor that promotes the highest EtBr accumulation in *S. aureus* [5].

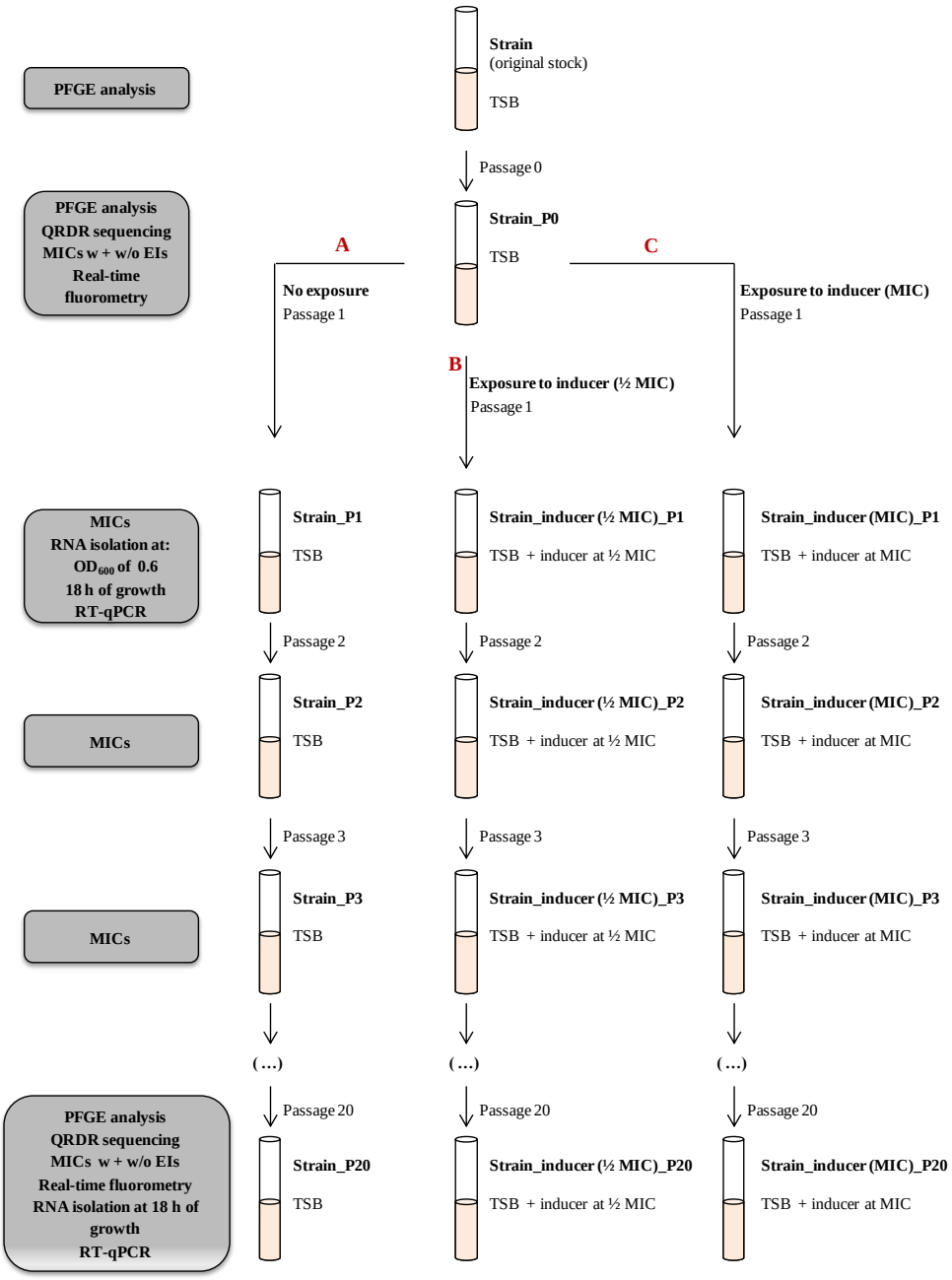

Figure 7.1. Diagram of the exposure processes to which the three strains in study were subjected. Each strain was subjected during 20 passages (days) after growth of the first culture (P1) to: (A) no exposure (drug-free media); (B) exposure to inducer at ½ its MIC; (C) exposure to inducer at its MIC. The inducers used were ethidium bromide (EtBr), ciprofloxacin (CIP) or cetrimide (CET). The concentration used for each exposure process considered the following MIC values: EtBr, 6.25 mg/L for ATCC25923, 8 mg/L for SM2 and SM50; CIP, 0.25 mg/L for ATCC25923, 32 mg/L for SM2 and 64 mg/L for SM50; CET, 2 mg/L for ATCC25923 and SM2, 4 mg/L for SM50. The several phenotypic and genotypic tests used to characterize each culture through the exposure process are indicated on the left. QRDR: quinolone-resistance determining region of genes *grlA* and *gyrA*; EI: efflux inhibitor.

After loading the cells with EtBr at 25°C for a period of 60 min, the cells were collected by centrifugation at 13,000 rpm for 10 min and re-suspended in 1X PBS to an OD_{600} of 0.6. Several parallel assays were then run in 0.1 mL final volume, corresponding to 0.05 mL of the EtBr-loaded cells incubated with 0.05 mL of (1) PBS 1X; (2) glucose 0.8%; (3) 400 mg/L verapamil; (4) glucose 0.8% plus 400 mg/L verapamil. The final concentrations were glucose 0.4%, 200 mg/L verapamil and OD_{600} of 0.3. The efflux assays were conducted in a Rotor-Gene 3000™ at 37°C, and the fluorescence of EtBr was measured (530/585 nm) at the end of every cycle of 10 sec, for a total period of 10 min. The raw data obtained was then normalized against data obtained from non-effluxing cells (cells from the control with only 200 mg/L verapamil), at each point, considering that these correspond to the maximum fluorescence values that can be obtained during the assay. The relative fluorescence thus corresponds to the ratio of fluorescence that remains per unit of time, relatively to the EtBr-loaded cells. The slope (m) of each resulting EtBr efflux curve was calculated by linear regression using the values obtained during the first 1 to 2 min of the assays, as they portray the linear behaviour of the EtBr extrusion from the cells. The Relative Index of Efflux activity (RIE) was also calculated using the following formula:

$$\frac{}{}$$

Where RF is the relative fluorescence value corresponding to the 10^{th} minute of the efflux assay, P0 corresponds to day 0 of exposure and P20 corresponds to day 20 of exposure. The determination of the RIE values for each exposure condition allows the direct comparison of the EtBr efflux activity of each strain after exposure to the efflux inducers to their initial condition. A RIE value of 0 corresponds to an efflux activity equivalent to that one present at the beginning of exposure. The maximum value of 1 corresponds to a 100% increase of EtBr efflux activity after the exposure process. Negative RIE values represent loss of EtBr efflux activity in comparison with the original efflux activity.

Gene expression analysis. The expression of the multidrug efflux pump genes *norA, norB, norC, mepA, mdeA* and of the regulators *mgrA* and *mepR* was evaluated at the time points mentioned previously (Figure 7.1). Total RNA isolation was performed using the Trizol method [2] as described in section 2.1.2. of this Thesis. The RNA was

quantified in a NanoDrop 1000 (ThermoScientific, Madison, WI, USA) and all the RNA samples corresponding to the same time point for each strain were adjusted to the same concentration with RNase-free water. The RNA integrity was evaluated by a 1% agarose-2.2M formaldehyde gel electrophoresis. Quantitative RT-PCR (RT-qPCR) was performed using the QuantiTect SYBR Green RT-PCR Kit (QIAGEN). The primers used in these assays are described in Table 7.2. Relative gene expression of genes *norA*, *norB*, *norC*, *mepA*, *mdeA*, *mgrA* and *mepR* was assessed by comparison of the relative quantity of the respective mRNA in the presence of efflux inducer to the control (inducer-free condition of the time point in analysis) by the comparative threshold cycle (C_T) method [20], using the *gyrB* gene as reference, in a Rotor-Gene 3000™ thermocycler with real-time analysis software. Negative controls and genomic DNA contamination controls were included. Genes showing increased expression of at least two-fold when compared to the control were considered to be overexpressed. In order to verify for the reaction specificity, a melting curve analysis was done after each assay and the RT-qPCR products were visualized by electrophoresis in 2% agarose gels.

Isolation of genomic DNA. Genomic DNA was isolated with the QIAamp DNA Mini Kit (QIAGEN), with an additional step of 30 min digestion with 0.2 mg/L of lysostaphin (Sigma) prior to extraction.

Screening of mutations in *grlA* and *gyrA* genes. Internal fragments comprising the QRDR of *grlA* and *gyrA* genes (corresponding to residues 32-177) were amplified using the primers described in Table 7.2. The reaction mixture (0.05 mL) contained 2.5 U of Taq Polymerase (Fermentas Inc., Ontario, Canada), 1X Taq buffer (Fermentas); 25 pmol of each primer; 0.2 mM of dNTP and 1.75 mM of $MgCl_2$. The amplification conditions were as follows: DNA was denatured at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 52 °C for 1 min and extension at 72 °C for 1 min, followed by a step of final extension at 72 °C for 5 min. Amplification products were purified and sequenced in both strands using the same set of primers. Sequences were analyzed and aligned using the freeware programs BioEdit and ClustalW, respectively.

Table 7.2. List of primers used in this study.

Primer	Sequence (5'-3')	Amplicon Size (bp)	Reference
For RT-qPCR experiments			
norA_Fw	TTCACCAAGCCATCAAAAAG	95	[5]
norA_RT(Rv)	CCATAAATCCACCAATCCC		
norB_Fw	AGCGCGTTGTCTATCTTTCC	213	[5]
norB_Rv	GCAGGTGGTCTTGCTGATAA		
norC_Fw	AATGGGTTCTAAGCGACCAA	216	[5]
norC_Rv	ATACCTGAAGCAACGCCAAC		
mepA_Fw	TGCTGCTGCTCTGTTCTTTA	198	[5]
mepA_Rv	GCGAAGTTTCCATAATGTGC		
mdeA_Fw	GTTTATGCGATTCGAATGGTTGGT	155	[5]
mdeA_Rv	AATTAATGCAGCTGTTCCGATAGA		
mgrA_Fw	GGGATGAATCTCCTGTAAACG	131	This study
mgrA_Rv	TTGATCGACTTCGGAACG		
mepR_Fw	TCGATGCACAAGATACGAGA	111	This study
mepR_Rv	GCGATACGAGTGTTTGTTCC		
gyrB_Fw	CGTAAATCAGCGTTAGATG	277	[25]
gyrB_Rv	TCGCTAGATCAAAGTCGCCA		
For mutation screening			
GrlA_Fw	AGGTGATCGCTTTGGAAGA	501	This study
GrlA_Rv	TGGTGGTATATCTGTCGCGTA		
GyrA_Fw	CCAGTGAAATGCGTGAATC	514	This study
GyrA_Rv	TGTGGTGGAATATTCGTTGC		

Fw: forward; Rv: reverse; bp: base pair.

7.3 Results

7.3.1. Evolution of the susceptibility profile throughout the exposure processes

The three strains in study were serially subjected, during a 20-day period, to an exposure process to sub-inhibitory (½ the MIC) or inhibitory concentration (MIC) of three known substrates of multidrug efflux pumps: EtBr, CIP and CET. The eventual occurrence of contamination during the exposure processes was ruled out by analysis of the SmaI macrorestriction profiles of the strains at the beginning (day 0) and the end (day 20) of each exposure process (Figure 7.2). The macrorestriction profiles of the strains remained unaltered, with the exception of strain SM50_EtBr(½MIC), for which is visible the disappearance of a ca. 291 kb band and the appearance of two bands with ca. 194 kb and 97 kb (Figure 7.2), which should be the result of a single genetic event, most probably the gain of a SmaI restriction site.

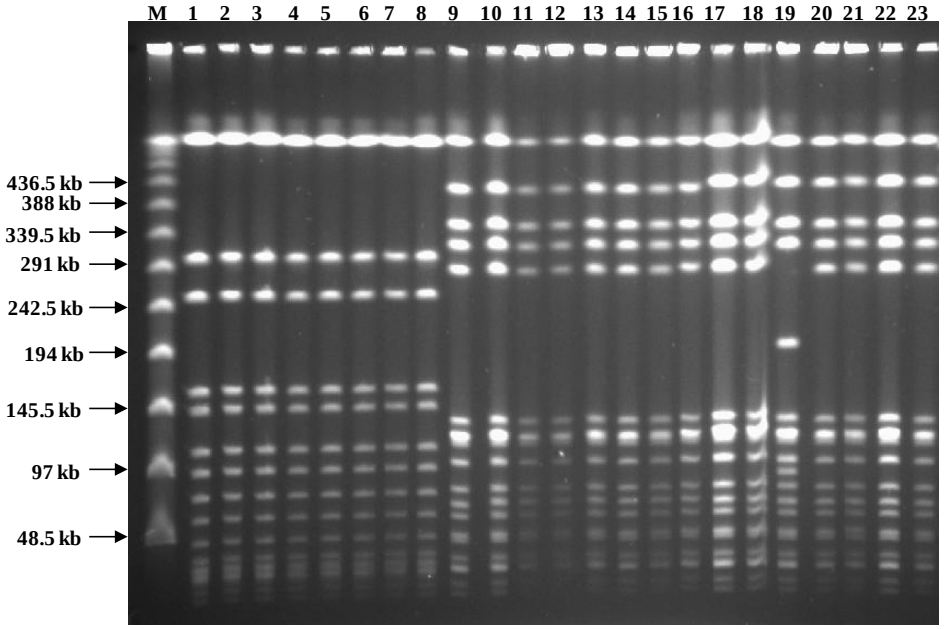

Figure 7.2. SmaI macrorestriction profiles of the three *S. aureus* strains in study at the beginning and at the end of each exposure process. M – "lambda Ladder PFG marker"; **1-** ATCC25923_original; **2-** ATCC25923_P0 **3-** ATCC25923_EtBr(½MIC)_P20; **4-** ATCC25923_EtBr(MIC)_P20; **5-** ATCC25923_CIP(½MIC)_P20; **6-** ATCC25923_CIP(MIC)_P20; **7-** ATCC25923_CET(½MIC)_P20; **8-** ATCC25923_CET(MIC)_P20; **9-** SM2_original; **10-** SM2_P0; **11-** SM2_EtBr(½MIC)_P20; **12-** SM2_EtBr(MIC)_P20; **13-** SM2_CIP(½MIC)_P20; **14-** SM2_CIP(MIC)_P20; **15-** SM2_CET(½MIC)_P20; **16-** SM2_CET(MIC)_P20; **17-** SM50_original; **18-** SM50_P0; **19-** SM50_EtBr(½MIC)_P20; **20-** SM50_EtBr(MIC)_P20; **21-** SM50_CIP(½MIC)_P20; **22-** SM50_CIP(MIC)_P20; **23-** SM50_CET(½MIC)_P20.

The evolution of the susceptibility profile of the strains was monitored by determination of the MIC values of the efflux inducers throughout the time-frame of the exposure processes (Figure 7.3, Tables 7.3-7.5). Overall and comparing the final MIC values obtained, an increase of two-fold to 16-fold in the MICs of the inducers was observed after the exposure processes for the three strains. Exposure to the highest inhibitory concentration did not always result in higher levels of resistance, as observed for ATCC25923 exposed to ciprofloxacin (Figure 7.3-B). The strains were less responsive to cetrimide (mostly MIC increases of none to two-fold) than to EtBr (MIC increases of two to 16-fold) or ciprofloxacin (MIC increases of two to eight-fold) (Figure 7.3-C). Also, no growth occurred for strain SM50 in the presence of the inhibitory concentration of cetrimide. In general, the strains presented differences in the response to the efflux inducers. The clinical strain SM2, with basal efflux activity, was the most responsive strain, showing the highest MIC increases. Conversely, SM50, the clinical strain that initially presented increased efflux activity, was the less responsive strain, presenting none to eight-fold increases in the MICs of EtBr, CIP or CET. Surprisingly, for SM50 in some exposure conditions, the final MIC values obtained were higher in the presence of the sub-inhibitory concentration (Figure 7.3-A and B). The reference strain ATCC25923, with basal efflux activity, showed a behavior similar to the clinical strain SM2.

The monitoring of the MIC values of the efflux inducers during the first three days of the exposure process revealed that the increases registered in the MIC values occurred, in most cases, in the first days of exposure. Besides, in some conditions this increase took place more swiftly in the presence of the inhibitory concentration of the inducer being tested, as in the case of the MICs for ATCC25923 exposed to EtBr (Figure 7.3-A).

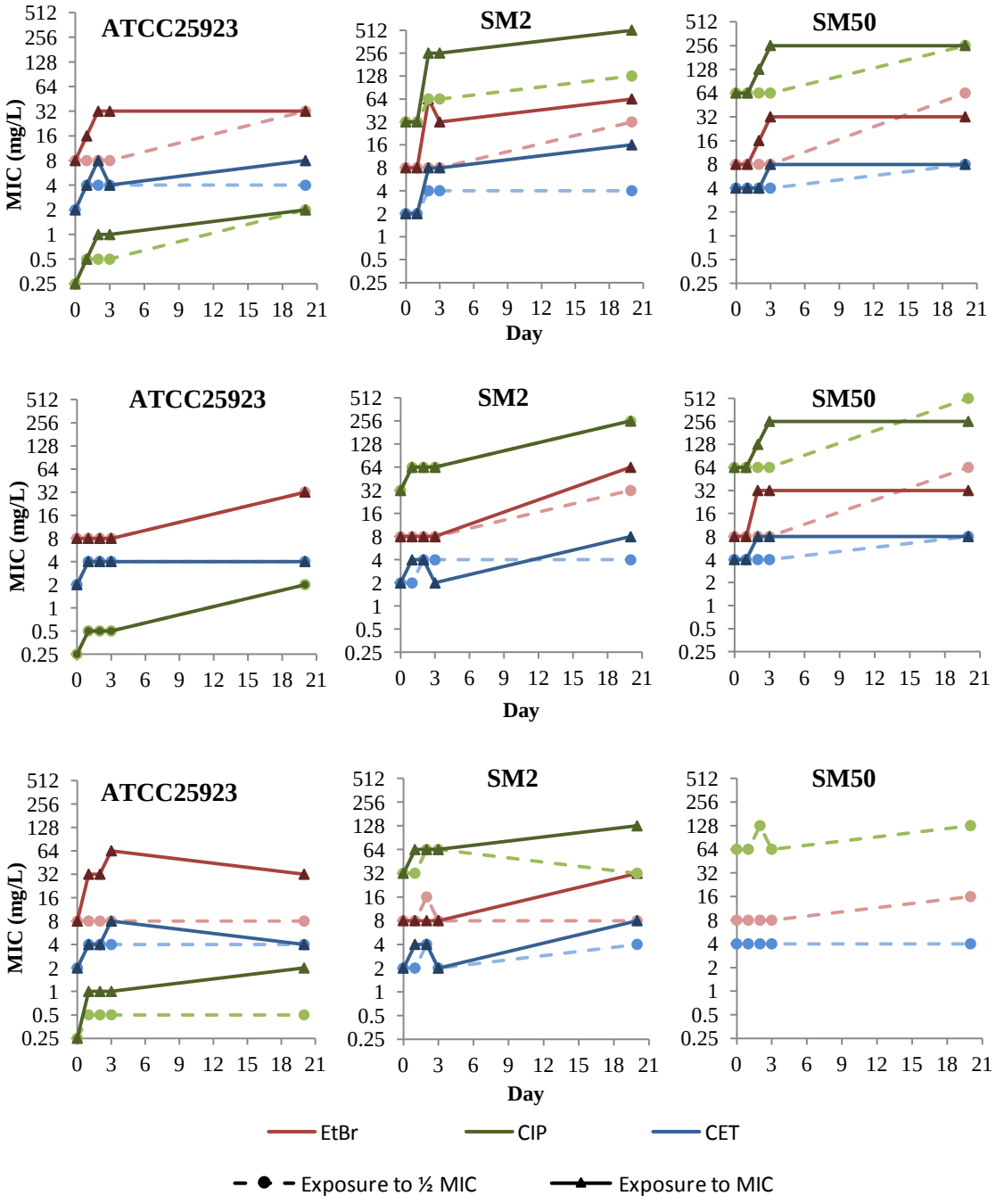

Figure 7.3. Evolution of the MIC values (mg/L) of ethidium bromide (EtBr, red), ciprofloxacin (CIP, green) and cetrimide (CET, blue) for the three strains in study throughout the 20-day exposure to ethidium bromide (A), ciprofloxacin (B) and cetrimide (C) at ½ the MIC (dotted lines) or at the MIC (full lines) concentrations (data available for days 0, 1, 2, 3 and 20th). No growth was obtained for strain SM50 at the CET MIC concentration.

One of the most stricking observations taken from the data depicted in Figure 7.3 and Tables 7.3-7.5 was that independently of the efflux inducer used or the concentration to which the strains were exposed to (less evident for the sub-inhibitory

concentration of cetrimide), similar final values of MICs were reached for each strain not only for the efflux inducer used during the exposure process but also for the remaining two efflux inducers tested (cf. panels A, B and C of Figure 7.3 and Tables 7.3-7.5). The three strains presented after exposure, irrespective of the inducer compound, EtBr MICs of 32 to 64 mg/L and CET MICs of 4 to 8 mg/L. In the particular case of CIP, for which the strains differed in the starting point of susceptibility, ATCC25923 presented a CIP MIC of 2 mg/L after most of the exposure processes. This particular value corresponds, according to CLSI guidelines, to intermediate resistance to ciprofloxacin [4], revealing that the initially susceptible strain ATCC25923 became resistant to fluoroquinolones as a response to the stress imposed not only by ciprofloxacin but also to non-fluoroquinolone antimicrobial compounds. Clearly, a multidrug efflux response, which was independent of the efflux inducer used, can be perceived for all strains subjected to exposure processes. The susceptibility profile of the strains towards other antibiotics and biocides was assessed at the beginning (day 1) and at the end (day 20) of each exposure condition for each strain (Tables 7.3–7.5). Regarding antibiotics, significant alterations in the MIC values were only detected for other fluoroquinolones, namely norfloxacin, with increases in the MICs of up to 16-fold, and in a lesser extent to levofloxacin. As was observed for CIP, ATCC25923 presented intermediate resistance to norfloxacin (MIC $\geq$ 8 mg/L), after the majority of the exposure processes and to levofloxacin (MIC = 1 mg/L), when challenged with the inhibitory concentration of CIP. No significant alteration in the susceptibility level was found for other antibiotics tested, namely, oxacillin, penicillin, vancomycin, chloramphenicol, tetracycline and gentamicin (data not shown).

Susceptibility testing towards several other biocides revealed increases in the MICs of all the compounds tested, including the quaternary ammonium compounds cetylpyridinium chloride, tetraphenylphosphonium bromide, benzalkonium chloride and dequalinium chloride, the diamidine pentamidine, and the bisbiguanidine chlorhexidine. These increases were higher, up to eight-fold, for the several quaternary ammonium compounds tested (excluding benzalkonium chloride) and for pentamidine and less evident (up to four-fold) for chlorhexidine, particularly in the diacetate form (Tables 7.3–7.5).

Table 7.3. MIC values (mg/L) of antibiotics, biocides and dyes for the strain ATCC25923 at the beginning and at the end of the exposure to ethidium bromide, ciprofloxacin and cetrimide. The numbers in brackets indicate the increase of the MIC values registered at the end of each exposure process.

	Original MIC	MIC after exposure to:					
		EtBr		CIP		CET	
		½ MIC	MIC	½ MIC	MIC	½ MIC	MIC
ATCC25923							
EtBr	8	32 (↑4x)	32 (↑4x)	32 (↑4x)	32 (↑4x)	8 (-)	32 (↑4x)
CIP	0.25	2 (↑8x)	2 (↑8x)	2 (↑8x)	2 (↑8x)	0.5 (↑2x)	2 (↑8x)
CET	2	4 (↑2x)	8 (↑4x)	4 (↑2x)	4 (↑2x)	4 (↑2x)	4 (↑2x)
NOR	0.5	8 (↑16x)	8 (↑16x)	>8 (>↑16x)	>8 (>↑16x)	2 (↑4x)	8 (↑16x)
LEV	0.25	0.5 (↑2x)	0.5 (↑2x)	0.5 (↑2x)	1 (↑4x)	0.25 (-)	0.5 (↑2x)
PT	32	128 (↑4x)	128 (↑4x)	64 (↑2x)	32 (-)	32 (-)	128 (↑4x)
CPC	0.5	1 (↑2x)	2 (↑4x)	2 (↑4x)	1 (↑2x)	2 (↑4x)	2 (↑4x)
BAC	2	2 (-)	2 (-)	2 (-)	4 (↑2x)	2 (-)	2 (-)
TPP	16	64 (↑4x)	64 (↑4x)	64 (↑4x)	16 (-)	16 (-)	32 (↑2x)
CHX	1	2 (↑2x)	2 (↑2x)	2 (↑2x)	1 (-)	2 (↑2x)	1 (-)
CHXg[a]	3E-5	6E-5 (↑2x)	12.5E-5 (↑4x)	6E-5 (↑2x)	6E-5 (↑2x)	6E-5 (↑2x)	6E-5 (↑2x)
DQ	4	8 (↑2x)	16 (↑4x)	8 (↑2x)	4 (-)	4 (-)	8 (↑2x)

EtBr: ethidium bromide; CIP: ciprofloxacin; CET: cetrimide; NOR: norfloxacin; LEV: levofloxacin; PT: pentamidine; CPC: cetylpyridinium chloride; BAC: benzalkonium chloride; TPP: tetraphenylphosphonium bromide; CHX: chlorhexidine diacetate; CHXg: chlorhexidine digluconate; DQ: dequalinium chloride. [a] The MIC values of chlorhexidine digluconate are given in % (p/v).

Table 7.4. MIC values (mg/L) of antibiotics, biocides and dyes for the strain SM2 at the beginning and at the end of the exposure to ethidium bromide, ciprofloxacin and cetrimide. The numbers in brackets indicate the increase of the MIC values registered at the end of each exposure process.

	Original MIC	MIC after exposure to:					
		EtBr		CIP		CET	
		½ MIC	MIC	½ MIC	MIC	½ MIC	MIC
SM2							
EtBr	8	32 (↑4x)	64 (↑8x)	32 (↑4x)	64 (↑8x)	8 (-)	32 (↑4x)
CIP	32	128 (↑4x)	512 (↑16x)	256 (↑8x)	256 (↑8x)	32 (-)	128 (↑4x)
CET	2	4 (↑2x)	8 (↑4x)	4 (↑2x)	8 (↑4x)	4 (↑2x)	8 (↑4x)
NOR	128	512 (↑4x)	>512 (>↑4x)	>512 (>↑4x)	>512 (>↑4x)	128 (-)	512 (↑4x)
LEV	32	32 (-)	64 (↑2x)	64 (↑2x)	64 (↑2x)	16 (↓2x)	32 (-)
PT	16	32 (↑2x)	64 (↑4x)	64 (↑4x)	64 (↑4x)	16 (-)	64 (↑4x)
CPC	0.5	2 (↑4x)	4 (↑8x)	2 (↑4x)	4 (↑8x)	0.5 (-)	2 (↑4x)
BAC	1	2 (↑2x)	4 (↑4x)	2 (↑2x)	2 (↑2x)	1 (-)	2 (↑2x)
TPP	32	64 (↑2x)	128 (↑4x)	128 (↑4x)	128 (↑4x)	32 (-)	64 (↑2x)
CHX	0.5	1 (↑2x)	1 (↑2x)	1 (↑2x)	1 (↑2x)	0.5 (-)	1 (↑2x)
CHXg[a]	6E-5	6E-5 (-)	12.5E-5 (↑2x)	12.5E-5 (↑2x)	12.5E-5 (↑2x)	6E-5 (-)	12.5E-5 (↑2x)
DQ	4	8 (↑2x)	16 (↑4x)	16 (↑4x)	16 (↑4x)	4 (-)	16 (↑4x)

EtBr: ethidium bromide; CIP: ciprofloxacin; CET: cetrimide; NOR: norfloxacin; LEV: levofloxacin; PT: pentamidine; CPC: cetylpyridinium chloride; BAC: benzalkonium chloride; TPP: tetraphenylphosphonium bromide; CHX: chlorhexidine diacetate; CHXg: chlorhexidine digluconate; DQ: dequalinium chloride. [a] The MIC values of chlorhexidine digluconate are given in % (p/v).

Table 7.5. MIC values (mg/L) of antibiotics, biocides and dyes for the strain SM50 at the beginning and at the end of the exposure to ethidium bromide, ciprofloxacin and cetrimide. The numbers in brackets indicate the increase of the MIC values registered at the end of each exposure process.

	Original MIC	MIC after exposure to:					
		EtBr		CIP		CET	
		½ MIC	MIC	½ MIC	MIC	½ MIC	MIC
SM50							
EtBr	8	64 (↑8x)	32 (↑4x)	64 (↑8x)	32 (↑4x)	16 (↑2x)	---
CIP	64	256 (↑4x)	256 (↑4x)	512 (↑8x)	256 (↑4x)	128 (↑2x)	---
CET	4	8 (↑2x)	8 (↑2x)	8 (↑2x)	8 (↑2x)	4 (-)	---
NOR	256	>512 (>↑2x)	>512 (>↑2x)	>512 (>↑2x)	>512 (>↑2x)	256 (-)	---
LEV	32	64 (↑2x)	32 (-)	64 (↑2x)	64 (↑2x)	32 (-)	---
PT	16	64 (↑4x)	64 (↑4x)	64 (↑4x)	32 (↑2x)	32 (↑2x)	---
CPC	1	2 (↑2x)	4 (↑4x)	2 (↑2x)	4 (↑4x)	1 (-)	---
BAC	2	4 (↑2x)	2 (-)	2 (-)	2 (-)	2 (-)	---
TPP	32	128 (↑4x)	128 (↑4x)	128 (↑4x)	128 (↑4x)	64 (↑2x)	---
CHX	1	1 (-)	1 (-)	1 (-)	1 (-)	1 (-)	---
CHXg[a]	6E-5	12.5E-5 (↑2x)	12.5E-5 (↑2x)	6E-5 (-)	12.5E-5 (↑2x)	6E-5 (-)	---
DQ	4	16 (↑4x)	16 (↑4x)	16 (↑4x)	16 (↑4x)	8 (↑2x)	---

EtBr: ethidium bromide; CIP: ciprofloxacin; CET: cetrimide; NOR: norfloxacin; LEV: levofloxacin; PT: pentamidine; CPC: cetylpyridinium chloride; BAC: benzalkonium chloride; TPP: tetraphenylphosphonium bromide; CHX: chlorhexidine diacetate; CHXg: chlorhexidine digluconate; DQ: dequalinium chloride. [a] The MIC values of chlorhexidine digluconate are given in % (p/v). --- Not applicable.

Thus, the results described so far indicate that challenging three *S. aureus* strains differing in their efflux activity with distinct substrates of multidrug efflux pumps promotes the development of a MDR phenotype. It is now essential to ascertain if these MDR phenotypes are associated with an increased efflux activity in these cells.

7.3.2. Assessment of efflux activity

The efflux activity was assessed by determination of the MIC values of the efflux inducers in the presence of the known efflux inhibitors thioridazine and verapamil (data not shown) (Table 7.6) and by fluorometric detection of EtBr efflux activity (Figure 7.4). Concerning the effect of efflux inhibitors on the initial MIC values, we had previously established that efflux contributes to the susceptibility levels of the reference strain ATCC25923 [8], SM2 and SM50 [5, 6], as demonstrated by the reductions in the efflux inducers MICs of up to eight-fold in the presence of the efflux inhibitors. This inhibitory effect is more evident in SM50 and was attributed to the higher efflux capacity of this strain [5, 6]. We now tested the effect of the efflux inhibitors for these strains at the end of each exposure process (day 20) (Table 7.6). A reduction in MICs of four-fold or higher in the presence of an efflux inhibitor when compared to the original values was considered as inhibition of increased efflux activity. As mentioned previously, the MICs of EtBr, CIP and CET increased for each strain after each exposure process. Overall, the inhibitors were able to reduce these MICs to values similar or lower than the original MICs (Table 7.6). This inhibitory effect was more pronounced for the strains exposed to EtBr, particularly ATCC25923 and SM2. This data indicates that the increase in resistance to the inducers and the other antimicrobial compounds promoted by the exposure to EtBr, CIP or CET is reversible and must probably results from an increase of the efflux activity in these three strains, which may be more pronounced for ATCC25923 and SM2, the strains with an initial lower efflux activity.

Table 7.6. Effect of the efflux inhibitor thioridazine (TZ) on the MIC values of the three efflux inducers at the beginning and at the end of exposure for the three strains in study.

	Original MIC (mg/L)		MIC (mg/L) after exposure to:											
			EtBr				CIP				CET			
			½ MIC		MIC		½ MIC		MIC		½ MIC		MIC	
	No EI	+ TZ	No EI	+ TZ	No EI	+ TZ	No EI	+ TZ	No EI	+ TZ	No EI	+ TZ	No EI	+ TZ
ATCC25923														
EtBr	8	1 (↓8x)	32	4 (↓8x)	32	1 (↓32x)	32	8 (↓4x)	32	4 (↓8x)	8	2 (↓4x)	32	4 (↓8x)
CIP	0.25	0.125 (↓2x)	2	0.25 (↓8x)	2	0.25 (↓8x)	2	0.5 (↓4x)	2	0.5 (↓4x)	0.5	0.25 (↓2x)	2	0.5 (↓4x)
CET	2	0.25 (↓8x)	4	0.5 (↓8x)	8	0.125 (↓64x)	4	0.5 (↓8x)	4	0.5 (↓8x)	4	0.5 (↓8x)	4	0.5 (↓8x)
SM2														
EtBr	8	2 (↓4x)	32	1 (↓32x)	64	2 (↓32x)	32	4 (↓8x)	32	2 (↓16x)	8	1 (↓8x)	32	4 (↓8x)
CIP	32	16 (↓2x)	128	16 (↓8x)	512	32 (↓16x)	256	32 (↓8x)	256	16 (↓16x)	32	8 (↓4x)	128	32 (↓4x)
CET	2	0.5 (↓4x)	4	0.25 (↓16x)	16	4 (↓4x)	4	2 (↓2x)	8	1 (↓8x)	4	0.5 (↓8x)	8	2 (↓4x)
SM50														
EtBr	8	1 (↓8x)	64	8 (↓8x)	32	8 (↓4x)	64	4 (↓16x)	32	4 (↓8x)	16	2 (↓8x)	---	---
CIP	64	16 (↓4x)	256	64 (↓4x)	256	64 (↓4x)	512	64 (↓8x)	256	64 (↓4x)	128	32 (↓4x)	---	---
CET	4	0.5 (↓8x)	8	1 (↓8x)	8	1 (↓8x)	8	2 (↓4x)	8	2 (↓4x)	4	1 (↓4x)	---	---

EtBr: ethidium bromide; CIP: ciprofloxacin; CET: cetrimide, EI: efflux inhibitor; TZ: thioridazine; ---: strain SM50 showed no growth in the presence of the CET MIC concentration. The values in brackets correspond to the decrease of the MICs in the presence of efflux inhibitors relatively to the original values (absence of efflux inhibitor); decreases of ≥ 4-fold are highlighted by the shadowed values.

To test this hypothesis, the efflux activity of each strain after the 20-day period of exposure to each condition was evaluated by real-time fluorometry (Figure 7.4). The slope of each EtBr efflux curve was determined in the first minutes (1-2) of the assays and relates to the rate of efflux. In addition, we also calculated the relative index of efflux activity (RIE), which corresponds to the cells overall capacity to efflux EtBr in the assays conditions. The EtBr efflux activity of each strain before exposure is represented in the three graphs (orange lines) of Figure 7.4 and confirms the basal efflux activity of both ATCC25923 and SM2 and the pronounced activity of SM50 (lower slope value) at day 0 of the exposure process. Assessment of efflux activity by this methodology after each exposure process revealed that the strains responded differently to the three inducers. Analysis of data presented in Figure 7.4 shows an increased efflux activity in all exposure conditions, except for the sub-inhibitory concentration of cetrimide for SM2 and SM50 and CIP for ATCC25923. In most cases, the highest the concentration to which a strain was exposed to, the highest the efflux activity detected (highest RIE values and lower slope values) (Figure 7.4). Interestingly, for the two clinical strains, exposure to either EtBr or ciprofloxacin resulted in comparable increased efflux of EtBr.

Altogether, this data confirms that the increased resistance towards different antimicrobials observed in all three strains at the end of the exposure process correlates with an augmented efflux capacity that was induced by the contact with substrates of multidrug efflux pumps. To ascertain which efflux pumps are activated and responsible for these MDR phenotypes, we then screened the expression levels of the genes coding for the main *S. aureus* MDR efflux pumps.

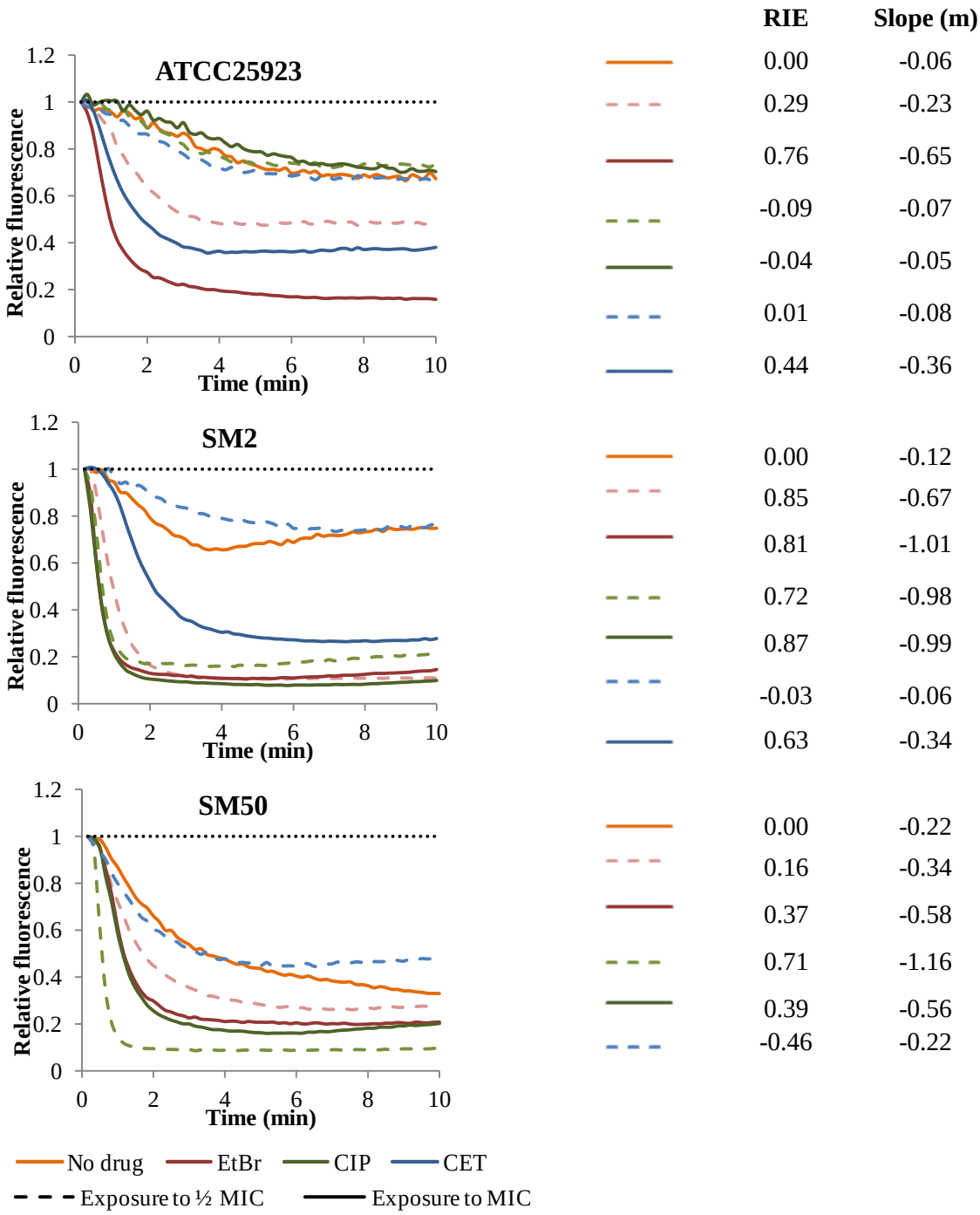

Figure 7.4. Assessment of EtBr efflux for the strains in study at the beginning (orange) and at the end of the exposure process to ethidium bromide (EtBr, red), ciprofloxacin (CIP, green) and cetrimide (CET, blue) at ½ the MIC (dashed lines) and at the MIC (full lines) concentrations. The assays were conducted in the presence of 0.4% glucose. The data presented was normalized against the data obtained in conditions of no efflux (dotted line, cells incubated without glucose in the presence of 200 mg/L of VER). RIE values were calculated as described in Material and Methods and allow the direct comparison of the EtBr efflux activity of the strain after exposure (P20) to their initial efflux activity (P0). The slope (m) of the EtBr efflux curves was calculated by a linear regression of the values obtained in the first minutes of the assay and it relates to the rate of EtBr efflux in each condition tested.

7.3.3. Evaluation of efflux pump gene expression levels

The level of expression of the MDR efflux pump genes *norA*, *norB*, *norC*, *mdeA* and *mepA* and of the regulator genes *mgrA* and *mepR* was assessed as described in the Material and Methods section. Briefly, gene expression was evaluated at different stages of the antimicrobials exposure process: an early response, throughout day 1 of exposure and a late response, at day 20 (Tables 7.7 – 7.9 and Figures S1-S3).

Exposure to EtBr. Table 7.7 and Figure S1 illustrate the genetic response of the three strains exposed to sub-inhibitory and inhibitory concentrations of EtBr. These data revealed a similar temporal pattern in the three strains for the expression of genes coding efflux-pumps that extrude EtBr. We detected in all strains, an early response, mediated by low overexpression levels of *nor* genes, namely *norB* in ATCC25923, *norA*/*norB*/*norC* in SM2 and *norA*/*norB* in SM50. This pattern changed, either by the reduction of expression levels and/or a shift in the genes overexpressed, after the 20-day exposure to EtBr, with a predominance of expression of the *mepA* gene and its regulator *mepR* (Table 7.7). This late and specific response via *mepA*/*mepR* genes was consistently verified at the sub-inhibitory concentration of EtBr for the three strains. However, at the inhibitory concentrations, only ATCC25923 showed overexpression of these two genes, whereas strains SM2 and SM50 overexpressed *norA* only. According to data on the evolution of the MICs and efflux activity, strain SM50 was less responsive to EtBr, presenting lower values of gene expression than ATCC25923 and SM2.

Table 7.7. Gene overexpression of MDR efflux pump genes and regulators by RT-qPCR of strains ATCC25923, SM2 and SM50 at different time points of exposure to EtBr.

	Time points	Gene overexpression after exposure to EtBr					
		ATCC25923		SM2		SM50[c]	
		½ MIC	MIC	½ MIC	MIC	½ MIC	MIC
Early response (day 1)	OD 0.6	-	-	***norC*** (2.65 ± 0.26)	***norC*** (8.24 ± 4.76)	***norB*** (4.57 ± 1.54)	-
	18 h	-	***norB*** (6.06 ± 3.07) ***mgrA*** (5.88 ± 2.25)	***norA*** (4.46 ± 0.65) ***norB*** (1.93 ± 0.09) ***norC*** (10.79 ± 3.13) ***mdeA*** (2.12 ± 0.71) ***mepA*** (8.12 ± 4.52) ***mepR*** (2.30 ± 0.00)	-	-	-
Late response (day 20)	18 h	***norB*** (1.94 ± 0.74) ***mepA*** (30.18 ± 7.07) ***mepR*** (29.00 ± 4.25)	***norB*** (2.77 ± 0.67) ***mepA*** (15.25 ± 4.42) ***mepR*** (20.41 ± 1.00)	***norB*** (2.93 ± 0.14) ***norC*** (2.08 ± 0.30) ***mepA*** (15.07 ± 2.94) ***mgrA*** (2.04 ± 0.59) ***mepR*** (34.65 ± 5.28)	***norA*** (5.89 ± 0.86)	***norA*** (3.62 ± 0.53) ***norB*** (2.56 ± 0.38) ***mepA*** (5.13 ± 0.75) ***mepR*** (5.91 ± 3.28)	***norA*** (7.25 ± 1.06)

Gene expression was measured in the presence of EtBr relatively to the inducer-free condition. The results are presented as the mean and standard deviation of at least two independent assays performed with extracted total RNA. Overexpression was considered for values ≥ 2.
(-): No gene overexpression detected (see also Figure S1).

Exposure to ciprofloxacin. Analysis of the gene expression data obtained with the strains under exposure to CIP revealed a very different genetic response (Table 7.8 and Figure S2). The first difference relies on the lower levels of gene expression promoted by ciprofloxacin, at either sub-inhibitory or inhibitory concentrations. Only ATCC25923 showed high levels of overexpression at the highest concentration of inducer. Secondly, the ciprofloxacin-susceptible strain ATCC25923 presented only an early response to the inhibitory concentration of ciprofloxacin, with all genes tested

being overexpressed, in particular *mepA* and *mepR* (Table 7.8 and Figure S2). On the other hand, the two ciprofloxacin-resistant strains SM2 and SM50, showed a predominance of *nor* genes overexpression in both early and late responses, with SM2 overexpressing *norA* and *norC* genes and SM50 overxpressing *norA* (early response) and *norB* plus *norC* (late response).

Table 7.8. Gene overexpression of MDR efflux pump genes and regulators by RT-qPCR of strains ATCC25923, SM2 and SM50 at different time points of exposure to CIP.

	Time points	Gene overexpression after exposure to CIP					
		ATCC25923		SM2		SM50[c]	
		½ MIC	MIC	½ MIC	MIC	½ MIC	MIC
Early response (day 1)	OD 0.6	-	-	***norC*** (4.45 ± 1.70)	***norA*** (3.16 ± 0.46)	***norA*** (5.74 ± 3.19)	-
	18 h	-	***norA*** (5.35 ± 2.29) ***norB*** (6.37 ± 1.55) ***norC*** (7.57 ± 3.96) ***mepA*** (30.41 ± 7.30) ***mdeA*** (2.45 ± 0.82) ***mgrA*** (5.86 ± 0.29) ***mepR*** (17.38 ± 7.43)	-	-	nd	***mdeA*** (4.24 ± 2.00)
Late response (day 20)	18 h	-	-	-	***norA*** (6.93 ± 2.33) ***norC*** (2.94 ± 0.43)	***norB*** (2.46 ± 0.00) ***norC*** (4.45 ± 2.29)	***norB*** (4.14 ± 0.20) ***norC*** (7.63 ± 2.21)

Gene expression was measured in the presence of CIP relatively to the inducer-free condition. The results are presented as the mean and standard deviation of at least two independent assays performed with extracted total RNA. Overexpression was considered for values ≥ 2.
(-): No gene overexpression detected (see also Figure S2); nd: not determined.

Exposure to cetrimide. The level of expression of the efflux pump genes and regulators for the three strains under exposure to cetrimide is depicted in Table 9 and in Figure S3. A different pattern of gene expression was observed for the three strains. Strain ATCC25923 presented a behavior similar to the one upon exposure to ciprofloxacin, with no alterations on gene expression in the presence of the sub-inhibitory concentration of cetrimide but showing a strong early response to the

inhibitory concentration, with high levels of overexpression for all the genes tested. The two clinical strains presented very different expression patterns. SM2 revealed a strong early response to both concentrations of cetrimide with overexpression of nearly all genes tested followed by a weak late response, mediated by *mdeA* or *norC* genes. Conversely, SM50 showed only a low level of overexpression of the *nor* genes either in the early (*norA*) or late (*norB* and *norC*) response to the sub-inhibitory concentration of cetrimide.

Table 7.9. Gene overexpression of MDR efflux pump genes and regulators by RT-qPCR of strains ATCC25923, SM2 and SM50 at different time points of exposure to CET.

	Time points	Gene overexpression after exposure to CET					
		ATCC25923		SM2		SM50[c]	
		½ MIC	MIC	½ MIC	MIC	½ MIC	MIC
Early response (day 1)	OD 0.6	-	-	***norA*** (4.87 ± 1.62) ***norC*** (4.63 ± 2.33) ***mepA*** (10.50 ± 3.53)	***norA*** (4.35 ± 1.71) ***norC*** (4.66 ± 1.99) ***mepA*** (15.64 ± 10.36) ***mepR*** (4.16 ± 0.61)	***norA*** (2.38 ± 0.12) ***mgrA*** (6.00 ± 2.83)	na
	18 h	-	***norA*** (7.83 ± 3.61) ***norB*** (45.40 ± 8.77) ***norC*** (39.17 ± 23.40) ***mepA*** (6.37 ± 1.55) ***mdeA*** (5.91 ± 3.77) ***mgrA*** (35.93 ± 15.56) ***mepR*** (24.12 ± 8.12)	***norA*** (4.51 ± 1.09) ***norB*** (2.16 ± 0.42) ***norC*** (21.97 ± 3.22) ***mepA*** (6.19 ± 1.80) ***mdeA*** (4.44 ± 0.22) ***mgrA*** (2.30 ± 0.00) ***mepR*** (3.22 ± 1.07)	***norA*** (4.44 ± 0.22) ***norC*** (3.61 ± 1.64) ***mepA*** (8.30 ± 4.27) ***mdeA*** (4.78 ± 0.70) ***mgrA*** (4.55 ± 2.41) ***mepR*** (4.45 ± 1.70)	-	na
Late response (day 20)	18 h	-	-	***mdeA*** (3.66 ± 0.89)	***norC*** (3.07 ± 0.93)	***norB*** (2.46 ± 0.00) ***norC*** (3.98 ± 1.34)	na

Gene expression was measured in the presence of CET relatively to the inducer-free condition. The results are presented as the mean and standard deviation of at least two independent assays performed with extracted total RNA. Overexpression was considered for values ≥ 2.
(-): No gene overexpression detected (see also Figure S3); na: not applicable.

7.3.4. Screening for the emergence of mutations associated with resistance to fluoroquinolones

The occurrence of mutations associated with resistance to fluoroquinolones was screened by analysis of the QRDR of the target genes of these antibiotics, *grlA* and *gyrA*, for the three strains exposed to both concentrations of ciprofloxacin. The ciprofloxacin-susceptible strain ATCC25923 presents a wild-type GyrA and a mutation in GrlA, Pro-144 → Ser, which was already detected in a few *S. aureus* isolates [19, 28] but not associated with resistance to fluoroquinolones, since isolates carrying this mutation show a CIP MIC of 0.25 mg/L [28]. Strain ATCC25923, with an original CIP MIC of 0.25 mg/L, gained no mutations throughout the exposure process to the sub-inhibitory concentration of ciprofloxacin. However, under exposure to the inhibitory concentration, ATCC25923 acquired the mutation S80F at the QRDR of GrlA, remaining non-mutated in GyrA. Screening of the *grlA* QRDR of this strain in intermediate stages of the exposure process revealed that this mutation was acquired at day 4 or 5 of exposure. The mutation S80F is one of the most common mutations ascribed to GrlA in *S. aureus* clinical isolates [14] and has been associated with resistance levels to ciprofloxacin that vary between 2 mg/L [28] and 3-12.5 mg/L [35], corresponding to resistance according to CLSI and EUCAST guidelines [4, 32]. Thus, the CIP MIC of 2 mg/L presented by ATCC25923 exposed to the inhibitory concentration of CIP is in accordance with the values described in literature. As mentioned previously, after all exposure processes (except for the sub-inhibitory concentration of CET), ATCC25923 presented a final CIP MIC of 2 mg/L. Thus, the QRDR of *grlA*/*gyrA* genes were also screened for these exposure conditions, but no occurrence of mutations was detected, indicating that the increase in resistance was due to increased efflux solely.

The ciprofloxacin-resistant strains SM50 and SM2 already carried, by the time of their isolation, a double mutation in GrlA (S80F/E84K) accompanied by a single mutation in GyrA (S84L). This combination of mutations has been associated with high level resistance to ciprofloxacin, with MICs of 100 to > 800 mg/L [35] and the same was verified for these strains. The QRDR region of the *grlA* and *gyrA* genes was also sequenced for these strains after ciprofloxacin exposure, but no additional mutations

were detected. This indicates that the level of increased resistance to fluoroquinolones presented by these strains after the exposure process (increases in the CIP MICs from 32 to 256 mg/L for SM2 and from 64 to 512 mg/L for SM50 and in NOR MICs from 128 to >512 mg/L for SM2 and from 256 to >512 mg/L for SM50, Tables 7.4 and 7.5) is solely attributable to increased efflux activity, as confirmed by data with efflux inhibitors. Screening for mutations in the strains exposed to EtBr and CET also showed that no mutations occurred during those exposure processes.

7.4 Discussion

Previous studies have shown that exposure to biocides and dyes could trigger an efflux-mediated response that resulted in reduced susceptibility to other biocides [16] and to fluoroquinolones [8]. It has also been demonstrated that efflux is an important component of resistance to these antimicrobial compounds in *S. aureus* clinical isolates [5, 6, 9]. In the present work, we further explored the efflux-mediated response of *S. aureus* to different antimicrobial compounds, not only over different concentration ranges of the inducers but also over different times of exposure to these agents. This approach may provide a better understanding of the contribution of each multidrug efflux pump to resistance and further insights in the process of development of resistance to antimicrobials in *S. aureus* and its relation with mutation-based resistance. We exposed three strains, differing in their efflux capacity and susceptibility to antimicrobials, to constant inhibitory and sub-inhibitory concentrations of three distinct agents and analysed the bacterial response to these agents, in particular the relationship between efflux pump genes expression, efflux activity and acquisition of mutations throughout the time of exposure. All these factors were correlated with the evolution of the susceptibility profile of the exposed strains. The agents selected were the fluoroquinolone ciprofloxacin, the biocide cetrimide and the dye ethidium bromide, all known substrates of the main *S. aureus* chromosomal multidrug efflux pumps.

Exposure to antimicrobial agents promotes the emergence of a MDR phenotype. Exposure to the selected antimicrobial agents during a 20-day period originated strains with increased resistance to these efflux inducer agents as well as cross-resistance to other antimicrobial agents, thus resulting in a multidrug resistance phenotype. This phenotype emerged whatever the inducer, its concentration and the strain initial susceptibility phenotype, and similar final levels of resistance were attained for each antimicrobial in each strain. Even so, higher (inhibitory) concentrations of inducer promoted an earlier rise of resistance (Figure 7.3) in some of the conditions tested.

The three strains presented, at the end of each exposure period, increased resistance to the dye ethidium bromide, the fluoroquinolones ciprofloxacin and norfloxacin, to the biocides cetrimide, pentamidine, cetylpyridinium chloride, tetraphenylphosphonium bromide and dequalinium chloride, with MIC values augmented by 2- to 16-fold. The level of resistance to the biocides benzalkonium chloride, chlorhexidine diacetate, chlorhexidine digluconate and to the more hydrophobic fluoroquinolone levofloxacin also increased but in a lesser extent (up to a 4-fold increase in the MICs). Other antibiotics were tested, including oxacillin, tetracycline and gentamicin, but no alterations occurred in their MICs during the exposure processes. The emergence of an efflux-mediated MDR phenotype upon exposure to antimicrobial agents has been observed in previous studies [8, 16], showing that exposure to a substrate of a multidrug efflux pump promotes cross-resistance to other substrates of the same pump. One of the most important findings of this work was that the challenge of a susceptible strain, ATCC25923, to a constant sub-inhibitory or inhibitory concentration of the dye EtBr or the biocide CET (only at inhibitory concentration), promoted crossed resistance to fluoroquinolones, revealing the potential role of these agents as a selective pressure for the emergence of resistance to fluoroquinolones in healthcare environments.

Emergence of the MDR phenotype was accompanied by increased efflux activity. The presence of increased efflux activity in the strains exposed to antimicrobials was established by determination of MICs in the presence of known efflux inhibitors of *S. aureus* and real-time fluorometry. Both approaches confirmed a higher efflux activity for the exposed strains and that the strains with an initial basal efflux activity,

ATCC25923 and SM2, were the ones showing a higher increase of this activity. MIC data with efflux inhibitors allowed to additionally establish the level of resistance that can be attributed to this increased efflux. Thus, the sum of the data gathered by the different methodologies correlates the emergence of the MDR phenotype with an augmented activity of efflux pumps.

Patterns of overexpression of multidrug efflux pump genes. The response of the strains to the different efflux inducers was further assayed by measuring the expression level of the genes coding for the main *S. aureus* multidrug efflux pumps, *norA/B/C*, *mepA* and *mdeA*, and the regulators *mgrA* and *mepR* at different time points; during the first day of exposure (mid-exponential growth phase, OD_{600} of 0.6, and 18^{th} hour of growth) and at the end of the exposure (20^{th} day of exposure). Taken as a whole, the overexpression pattern found suggests a temporal differentiation in the activation of multidrug efflux pump genes (cf. Tables 7.7-7.9). The response of the strains to the inducers could be distinguished in two phases, an early-response, occurring throughout day 1 of exposure, where the strains responded strongly and in a non-specific manner by overexpressing several efflux pump genes, and a more specific late-response, that occurred with the prolonged exposure to the stimulus, in which the number of efflux pump genes overexpressed and/or their level of expression decreased. This reduction in expression levels may occur because the number of efflux pump proteins in the cell membrane required to cope with the antimicrobial compounds may have reached a maximum value, suggesting that the reduced expression level observed at day 20 may be the necessary to compensate for the protein turnover rate.

In a closer look and comparing the response of each strain to each inducer, different patterns of gene expression were found. A predominance of expression of some genes was detected under exposure to EtBr and CIP, namely the *mepA* gene for EtBr and the *nor* genes for CIP. These observations are in accordance with the substrate profile described for these multidrug efflux pumps, as EtBr is a good substrate of MepA and fluoroquinolones are a common substrate of the NorA, NorB and NorC pumps [7]. Regarding CET, a preferable expression pattern of efflux pump genes could not be established since a strong response with several genes was observed, except for SM50

that overexpressed the *nor* genes. This non-specific response to CET may reflect the different, broader effect that this membrane-active compound exerts upon the bacterial cell [17] that triggers a global stress response. Regarding the concentration of the stimulus, a correlation between inducer concentration and gene overexpression was only observed for strain ATCC25923, which was independent of the inducer. For neither the clinical strains SM2 nor SM50, an increasing concentration of efflux inducer resulted in a stronger/earlier response.

Overall, we observed for the three strains tested, an efflux-mediated response to the inducers via increased MIC values, which could be reduced by efflux inhibitors, increased EtBr efflux activity and overexpression of efflux pump genes. Noteworthy, this overall response was less pronounced for SM50, the only strain which already showed an increased efflux activity at the beginning of the exposure processes. This difference translates in the different behaviour of both clinical strains in response to the EtBr and CET stimulus. While strain SM2 presented a strong early response with overexpression of several efflux pump genes, the response of SM50 was weaker (cf. Tables 7.7 and 7.9). Regarding the response to CIP, both strains showed mild values of gene overexpression, which may be correlated to the presence of the QRDR mutations that these strains carry and already confer high-level resistance to fluoroquinolones. Thus, the mild gene overexpression in the presence of ciprofloxacin may account for the additional level of resistance due to efflux. Previous data suggested that clinical strains are primed to efflux noxious compounds, a trait that may be attributable to their prior exposure to antimicrobials in the hospital environment [5]. The different behavior of both strains towards the different stimuli is particularly striking as these are very closely related, as demonstrated by PFGE analysis and illustrates the multiple efflux-mediated responses that *S. aureus* can display.

SM50 requires the activation of further efflux pump proteins to cope with the stress stimuli and/or the production of extra efflux pump proteins to account for protein turnover. For SM2, the gene expression levels increased during day 1 of exposure, suggesting that the initial number of efflux pump proteins in the cell membrane was not sufficient to manage the presence of the noxious compounds. The susceptible reference strain ATCC25923, with an initial basal efflux activity, presented behaviour similar to SM2, with strong responses to all three compounds. Following the same reasoning, this

is a prototype strain with no prior exposure o antimicrobials, thus it is not primed for efflux requiring higher levels of efflux pump gene expression to cope with the noxious stimulus. However, some differences were detected from previous results for this strain. One of the unexpected results was the specific response to EtBr with expression of the MepA efflux pump. In a previous work, exposure of this strain to EtBr had triggered a preferred induction of the *norA* gene [8]. This difference could be due to the different approach used, as in the present study the strain has been subjected to a constant concentration of EtBr, either sub-inhibitory or inhibitory, whilst in the previous work the concentration of the stimulus was inhibitory and increased over time. This premise supports previous findings suggesting that the same strain may respond to the same inducer via different efflux systems, depending on the concentration of the inducer and the time of contact with the inducer, being an example of the multitude of efflux-mediated responses that can occur in *S. aureus*.

Efflux as a contributor to early resistance to fluoroquinolones. The results on the evolution of the susceptibility profile of the exposed strains and gene expression analysis demonstrated that efflux is a first-line response in resistance to three different types of antimicrobials for the three strains in study. In the particular case of the clinical strains, it could be demonstrated that efflux is an important component of resistance as the ciprofloxacin-resistant clinical strains, harboring known fluoroquinolone resistance mutations at the beginning of the exposure process, did not acquire additional mutations upon a continuous stimuli but instead suffered a significant increase in their efflux activity, especially SM2. As for the fully susceptible reference strain, this study demonstrated that efflux was the first response to ciprofloxacin exposure, being responsible for a level of resistance to this class of antibiotics that corresponds to clinically-significant resistance. It was also shown that, in the presence of an inhibitory concentration of CIP, the acquisition of mutations associated with fluoroquinolone resistance follows the activation of efflux pumps, thus demonstrating in vitro the process we believe occurs in vivo, particularly, in the clinical environment. Moreover, this study demonstrated that this same resistance phenotype can be achieved by exposure to non-fluroquinolone efflux substrates such as the dye EtBr or the biocide

CET. These findings provide further evidence that efflux is an important player in the emergence of fluoroquinolone resistance in *S. aureus*.

7.5 Conclusions

The last decade witnessed a growing awareness of the clinical importance of efflux as a mechanism of resistance to antimicrobial compounds [26]. In *S. aureus*, reports of clinical isolates with an established multidrug resistance phenotype attributable to efflux activity were scarce, and associated efflux activity with low increases in the antibiotic MICs [23, 24, 28]. For these reasons, efflux-mediated resistance to antimicrobials has been considered as clinically non-relevant in *S. aureus* and consequently neglected in comparison with target-based mutations, which originate high-level resistance [15]. In recent years, a new perspective on the role played by multidrug efflux systems has been emphasized, which are now perceived as important players in the emergence of resistance in bacteria [12, 13]. Overexpression of multidrug efflux pump genes has been shown to occur frequently in *S. aureus* clinical isolates [9, 18] and to contribute to low-level resistance to fluoroquinolones and biocides [5, 6, 9]. The findings of this study show that multidrug efflux pumps substrates, including fluoroquinolones and biocides, compounds that are commonly used in the hospital environments, promote a physiological response by the *S. aureus* cells, which is based on efflux and that persists over time with the maintenance of the stimulus. This efflux-mediated response can trigger resistance to fluoroquinolones, even by non-fluoroquinolone compounds, in a step-wise manner; a first step with activation of multidrug efflux pump genes and a second step with acquisition of QRDR mutations. These are promising results, as they establish the key role of efflux in the first-line response to these antimicrobial agents and suggest that exposure to these agents may act as selective pressure for the maintenance and dissemination of fluoroquinolone resistance, which, in turn, has been presumed to act as a selective factor for the persistence of MRSA strains in the hospital.

7.6 References

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CHAPTER 8

General Discussion

The Gram-positive bacterium *Staphylococcus aureus* has been a predominant pathogen agent in hospitals for several decades. Introduction of penicillin into the clinical practice envisaged a new era for the treatment of staphylococcal infections, but soon *S. aureus* revealed a fascinating aspect of its biology, the notable ability to develop and acquire resistance to antimicrobial agents. Nowadays, *S. aureus* is a paradigm of how the selective pressure exerted by antibiotics and other antimicrobial compounds can render a susceptible bacteria into a well-established multidrug resistant pathogen. Understanding the underlying mechanisms of resistance is therefore of paramount relevance. In *S. aureus*, resistance to all major classes of antibiotics and other antimicrobial agents has been reported via all mechanisms known to date, including enzymatic degradation/modification of the antimicrobial, target alteration, decreased permeability of the cell wall and efflux of the antimicrobial agent [6]. The latter has recently gained more importance, with the recognition that many bacterial efflux pumps have the capacity to expel antimicrobial compounds from the cell that are structurally and chemically distinct, promoting the appearance of MDR phenotypes [50, 51].

It was the objective of this Thesis to address questions concerning the efflux-mediated resistance to antibiotics and biocides in *S. aureus*, namely a revision of the physiological role of some of the *S. aureus* MDR efflux pumps (Chapters 2-4), the efflux-mediated resistance to antimicrobial agents (Chapters 5-6), and finally to evaluate the role of efflux as a player in the emergence of resistance (Chapter 7). The work focused on a collection of 53 *S. aureus* clinical isolates collected at a Portuguese hospital, of which 52 were resistant to ciprofloxacin, and was complemented with the study of two prototype strains, *S. aureus* ATCC25923 and the MRSA strain HPV107. In this chapter, I will provide a exhaustive discussion of the results obtained in this Thesis.

Strategies for assessment of efflux activity and expression of efflux pump genes

To conduct studies on efflux-mediated resistance, one must possess the adequate tools to measure efflux activity. The methodologies usually used for this purpose rely on the determination of MICs in the presence of efflux inhibitors, determination of

MICs of the broad spectrum substrate ethidium bromide or measurement of efflux activity by fluorometry [14, 42, 47]. The design and optimization of protocols to access efflux activity has been one of the focal points of the work developed by the Group of Mycobacteriology of IHMT/UNL [68]. These methods use EtBr as a marker molecule based on its properties as a fluorophore and a common substrate of MDR efflux pumps. The EtBr-agar Cartwheel method, a protocol designed by the group, was first developed to assess efflux activity in prototype and laboratory-derived strains [37] and was proven successful when applied to large collections of bacterial clinical isolates, including *S. aureus* [38]. This methodology was chosen, in this Thesis, as the starting point to screen efflux activity amongst the collection of 53 *S. aureus* clinical isolates (Chapters 5 and 6). A second protocol also developed by the group, which is based on real-time fluorometric detection of EtBr accumulation/efflux, was then used to corroborate the previous selection of isolates with increased efflux activity (Chapters 5 and 6). The presence of efflux activity was also complemented by determination of MICs of efflux pump substrates in the presence of efflux inhibitors. In literature, the efflux inhibitor of choice for *S. aureus* is reserpine, but initial screening with this compound revealed that, adding to solubility problems, it is a relatively weak inhibitor (Chapter 3.1) [12], as demonstrated by others [18]. In our experience, compounds such as verapamil and the phenothiazines thioridazine and chlorpromazine, revealed to be more effective as inhibitors, and thus more reliable in the detection of efflux activity. The choice of this approach was proven consistent in the identification of strains with increased efflux activity, as observed in Chapters 5 and 6, and in the characterization of the efflux activity and its correlation with the antimicrobial resistance profiles presented by those isolates and the reference strains.

Once the methodology for evaluation of efflux activity in *S. aureus* clinical isolates was established, and increased efflux activity detected, we focused on the best approach to measure the expression level of efflux pump genes. Previous work from our team in *E. coli* and *S. aureus* revealed that the combined use of RT-qPCR methodology with analysis by the comparative threshold cycle (C_T) method was a powerful tool for the detection of overexpression of efflux pump genes in these bacterial strains [13, 66, 67]. However, in both studies, the gene expression levels were assayed for strains adapted to antimicrobial compounds, and the respective parental strains were used as

standards for comparison. In this Thesis, we aimed to detect increased expression levels of efflux pump genes in clinical isolates with increased efflux activity. Thus, we came across several issues concerning the best standard to use as reference values, (i) either a reference strain or the clinical isolate itself; (ii) the possible need to induce the expression of these genes and if so, which inducer to use at what concentration; and finally, (iii) the definition of the most suitable cut-off value for considering gene overexpression. A review of the scarce literature available at the time revealed that the approach usually taken was similar to ours, with the prototype strain *S. aureus* SH1000 being used as standard for the calculation of relative gene expression and using the cut-off value of 4 for overexpression [14]. Making use of this information, the first approach to measure efflux pump gene expression in our isolates relied on the use of the reference susceptible strain ATCC25923 as the standard for basal expression level. The data gathered by this strategy provided no insights on expression levels as no overexpression was detected in either efflux-positive or efflux-negative isolates tested. However, new studies contemporary to these were unraveling new data on the regulation pathways for some of these efflux pumps, showing that the genetic background of each strain could have significant implications on which efflux pumps were activated or repressed [62, 63]. Taking into consideration these results, our second approach was to assure that differences in expression were not due to different genetic backgrounds. Thus, we induced efflux pump gene expression by comparing the levels of expression in a given strain exposed to an inducer relatively to the levels detected for that strain itself in the absence of that inducer. The concentration chosen for this stimulus was a sub-inhibitory one, defined as ½ the MIC of the inducer for each strain. This approach also tried to simulate the environment to which the clinical isolates have been previously exposed in the hospital environment. The data obtained using this strategy allowed us to delineate a pattern of expression for both reference strain and clinical isolates, as described in Chapter 5.

In spite of promising results attained with this last strategy, the finding of consistently increased levels of expression for some genes, but not overexpression, was worrisome, as exemplified by the consistent increased expression of the *norA* gene in strain SM1 under exposure to ciprofloxacin (3.25 ± 0.00) (Chapter 2.2). To become more certain in our data, we analyzed all the steps involved in our experiments, and

decided to improve a major step, the isolation of total RNA by replacing the previous used commercial kit with extraction with Trizol, a reagent consisting in a solution of phenol and guanidine isothiocyanate, which facilitates the isolation of small and large RNA species and maintains the integrity of the RNA due to a highly effective inhibition of RNase activity while disrupting cells and dissolving cell components during sample homogenization [8]. The use of Trizol provided increased RNA yields and allowed an improved control of the quantities used in each assay, reinforcing the reliability of our data and allowing to lower the cut-off value in our gene expression assays, from four to two (Chapter 7). The data gathered with this new experimental approach proved to result in a consolidation of our gene expression assays, although the definition of the cut-off value for considering increased gene expression remains subjective and should be considered as such.

Evaluation of the inhibitory effect of efflux inhibitors

One of the objectives of this Thesis is the evaluation of efflux inhibitors on the reduction or reversal of resistance in *S. aureus*. The inhibitory activity of these compounds was tested on the inhibition of EtBr efflux activity assessed by fluorometry and on their ability to reduce MIC values of several substrates of *S. aureus* MDR efflux pumps.

Throughout the many assays conducted, several efflux inhibitors were tested, including CCCP, reserpine, phenothiazines and verapamil. Initial studies showed that among these compounds, CCCP had little or absent inhibitory effect at sub-inhibitory concentrations [13]. Although this compound had been frequently used in this type of studies, the concentration used in those earlier studies exceeded largely the inhibitory concentrations and were therefore not significant for assaying inhibition of efflux activity [13]. Reserpine was also found to be a weak efflux inhibitor in comparison with phenothiazines and verapamil. These two last compounds, in particular phenothiazines, were the most effective ones in reducing the MICs for the several substrates tested. Their effect upon representative ciprofloxacin-resistant clinical strains, all carrying QRDR mutations in GrlA and GyrA proteins, was discussed in Chapters 5 and 6.

Overall, we could observe that phenothiazines were able to promote a reduction of the MICs of fluoroquinolones, albeit with no reversal of the resistance phenotype (Chapter 5), with the exception of one isolate (Chapter 6). These results indicate that the use of efflux inhibitors as co-adjuvants in therapy could potentiate the reduction/reversal of resistance in *S. aureus*.

However, for their use in therapy it is necessary to have further insight into their mechanism of action. Although phenothiazines and verapamil were the most effective compounds, they differed in their activity. While verapamil was more efficient in the shorter fluorometry assays (60 min), phenothiazines showed higher effect upon the 18 h assays of MIC determination. This difference in inhibitory activity may be a direct result of the mechanism of action of each compound on the bacterial cell. Verapamil has been shown to inhibit efflux activity in several bacterial species, including *S. aureus* and mycobacteria [1, 52]. Its actual mechanism of action is not elucidated, but verapamil is a known blocker of calcium channels in eukaryotic cells and is thought to play a similar role in bacteria, blocking calcium transport at the membrane level and leading to the intracellular accumulation of noxious compounds [69]. In the case of phenothiazines, their activity as efflux inhibitors has been long recognized [13, 25] but their mechanism of action remains unclear. Traditionally, phenothiazines are described as blockers of the calcium channel of eukaryotic cells [69]. However, recent studies in *S. aureus* have shown that thioridazine affects the transcription of genes associated with the cell wall biosynthesis [5] and that it could influence the cell wall composition [61] and interfere with membrane-based processes, probably by embedding into the cytoplasmatic membrane [26, 61]. Altogether, these experimental evidences support different modes of action for these two classes of compounds, as the disturbance of the membrane polarization by verapamil could account for the immediate inhibitory effect of this compound, whereas the effect of phenothiazines upon the cell membrane would be slower and long-lasting. Nevertheless, further studies should be performed to clarify the mechanism(s) of action of these two classes of compounds to fully understand their effect as efflux inhibitors in bacteria.

Characterization of the physiological role of *S. aureus* multidrug efflux pumps

NorA

Part of the work developed during this Thesis focused on the clarification of the physiological role of three of the MDR efflux pumps already described for *S. aureus*, namely the chromosomally-encoded NorA and the plasmid-encoded QacA and Smr.

In the literature, NorA has been the prototype efflux pump used to study efflux-mediated resistance in *S. aureus*. Hence, it was chosen as the starting point of our studies. Continuing earlier studies on NorA and aiming to explore the mechanisms that modulate *norA* expression and NorA activity, we further studied this efflux pump on a defined genetic system previously developed [11, 13]. This system consists on the susceptible reference strain ATCC25923, its counterpart EtBr-adapted, $ATCC25923_{EtBr}$, and reverted $ATCC25923_{EtBr\_rev}$. The first finding of this work was the transient nature of the *S. aureus* response to the stress imposed by EtBr. Particularly, we demonstrated that the exposure to/withdrawal of EtBr, a common MDR efflux pump substrate, promoted an increase/decrease level of resistance to fluoroquinolones, biocides and dyes (Chapter 2.1). Moreover, the increased susceptibility to these compounds displayed by the reverted strain when compared to the original strain reinforces the inducibility of the adaptation/reversion process and hints on the existence of mechanisms to "switch-off" the efflux-mediated response when no longer needed. This efflux-mediated response had been previously attributed to an increased level of *norA* expression [13].

The work described in this Thesis complements previous data and shows that *norA* increased expression in the EtBr-adapted strain is accompanied by increased expression of the *mgrA* gene, coding for the global regulator MgrA, involved in the regulation of several efflux pump genes (see 1.4.3.1). It was also found that both genes suffered a reduction in expression in the reverted counterpart. These results demonstrated that the increased expression level of *norA* was not due solely to the

deletion found in the promoter region of the EtBr adapted strain. The fully reverted resistance phenotype displayed by the reverted strain, together with the maintenance of *norA* overexpression further suggests that the regulation of this efflux system could also take place at a post-transcriptional level, as observed in other studies [67].

Our experimental approach lead us to hypothesize that the prolonged exposure of the susceptible strain ATCC25923 to EtBr promoted an increased expression level of *norA* in ATCC25923$_{EtBr}$ that is the sum of the effects due to a de-repression by MgrA and occurrence of the deletion on the *norA* promoter region. With the withdrawal of the EtBr stimulus and the maintenance of *norA* expression in ATCC25923$_{EtBr_rev}$, mutations occurred in the structural component of *norA* that resulted in an altered NorA, with a probable reduction of activity. This last finding indicates that the maintenance of NorA overproduction may be deleterious to the cell, leading to the introduction of mutations that may cause a reduction in NorA activity. The noxious effect of efflux pump overproduction has been reported in other bacterial species [3], but a similar effect on *S. aureus* is still to be explored.

Our second approach to study the NorA efflux system consisted on the evaluation of the genetic variability of the *norA* gene in a group of ciprofloxacin-resistant clinical *S. aureus*, isolated in a Portuguese hospital (Chapter 2.2). Some studies have hypothesized if, in parallel to what is observed with the QacA/B efflux systems in which a single nucleotide difference is implicated in a distinction of substrate specificities for these two pumps, the genetic variability of *norA* could also have a similar impact on NorA activity [43, 56, 59]. A phylogenetic study of the *norA* gene from representative isolates of the 52 ciprofloxacin-resistant isolates and from completely sequenced *S. aureus* strains corroborated a division of the circulating *norA* alleles, *norAI* and *norAII*, in two phylogenetic clusters. Allele *norAI* and its variants were more frequent among the clinical isolates studied, in line with the findings from other studies [43, 56, 59] and as suggested by the phylogenetic tree obtained. The data presented in Chapter 2.2 suggests that the presence of a mutation in the *norAI* allele producing an altered NorA (Gy-147 → Ser) could potentially have a higher impact on the resistance levels towards fluoroquinolones and biocides. The occurrence of mutations/insertions/deletions in the *norA* promoter region has been linked to an increased level of expression of the gene [14, 23]. However, despite the finding of such

alterations in the majority of the strains studied, we were unable to correlate them with increased expression of the respective *norA* allele. In addition to these alterations in the promoter region, the majority of the strains tested also carried mutations in the structural gene that lead to the production of altered NorA pumps. Although a direct correlation between increased efflux and NorA activity could not be established, some of the results obtained were indicative of an unpredicted outcome of those mutations upon NorA activity, namely in terms of resistance to fluoroquinolones, ethidium bromide and some biocides, including benzalkonium chloride and pentamidine.

One of the main findings of this study was the high frequency of alterations found in the *norA* promoter and/or structural gene in *S. aureus* clinical strains. Most of these alterations were already described in prototype strains and clinical isolates [56, 27], reinforcing their potential role in the modulation of *norA* expression or NorA activity. This work also highlighted the need of expanding our knowledge on NorA, in particular, on its structure and topology. Conducting site-directed mutagenesis would be a valuable tool to ascertain the actual effects of the mutations found in NorA efflux activity. Another outcome of this study was the lack of correlation between *norA* promoter regions and increased efflux expression. Serving as a cautionary sign, this result proved important since the finding of these alterations in the promoter is being used in several current studies as a predictor of *norA*-efflux mediated resistance [19, 22, 44]. Moreover, as the data on the regulation of this efflux pump accumulates, we become more aware that several additional factors play major roles in the modulation of *norA* expression and NorA activity.

In conclusion, the data gathered in Chapter 2 showed that NorA is an important efflux system in the response of *S. aureus* to stress, and that its role may be more relevant in the presence of high concentrations and/or prolonged exposure to the chemical stress stimulus.

QacA and Smr

Plasmid-encoded efflux pumps are associated with resistance to several biocide compounds [51]. The contribution of QacA and Smr efflux pumps to resistance was

ascertained in three distinct MRSA clinical isolates and the plasmids carrying these determinants were characterized for two of those strains (Chapters 3 and 4). QacA efflux activity was correlated with increased resistance to quaternary ammonium compounds (the monovalent tetraphenylphosphonium bromide, cetrimide, cetylpyridinium chloride, benzalkonium chloride and the divalent dequalinium chloride), diamidines (pentamidine) and biguanidines (chlorhexidine) and to dyes (ethidium bromide and acriflavine). As expected, no correlation between QacA and antibiotic resistance was found. The increased resistance profile towards these QACs, diamidines, biguanidines and dyes, presented by the two strains carrying the *qacA* gene, HPV107 and SM39, corresponds to the substrate profile described for this pump [41, 49]. On the other hand, Smr efflux activity was associated with increased resistance to a narrower number of compounds that included the monovalent quaternary ammonium compounds cetrimide, cetylprydinium chloride and benzalkonium chloride and the dyes ethidium bromide and berberine. Moreover, the level of resistance conveyed by Smr was lower than the one conferred by QacA. In a recent study by Furi *et al.*, a large collection of *S. aureus* clinical isolates was screened for reduced susceptibility to biocides, in particular benzalkonium chloride and chlorhexidine, by determination of MICs and minimum bactericidal concentrations (MBCs). Although unable to identify a sub-population with reduced susceptibility to these agents, the study reports the finding of a correlation between the presence of *qac* genes, including *qacA* and *smr*, with an increase in mode MIC of the sub-populations carrying these determinants [19], reinforcing the need for better understanding the contribution of these efflux systems and the plasmids that carry them to resistance phenotypes in *S. aureus* clinical isolates.

The *qacA* gene detected in strain SM39 was harbored by a large (26 kb) non-conjugative multiresistance plasmid, designated pSM39, encoding additional determinants for resistance to β-lactams and heavy-metals. However, the *smr* gene, carried by strain SM52, was encountered on a small (2.8 kb) non-conjugative plasmid named pSM52 that carried no additional resistance genes. Recent large-scale investigations into the plasmid diversity of staphylococci showed that large non-conjugative multiresistance plasmids account for the majority of the plasmids described to date in *S. aureus* and other coagulase-negative staphylococci [57]. This finding suggests that selective pressure exerted by antimicrobials may be a major driving force

for the maintenance and dissemination of these plasmids, inasmuch as the resistance to antimicrobials used in different applications favor *S. aureus* survival in distinct environments. For instance, tobacco use and occupational hazard have been proposed to be linked to cadmium resistance for which the resistance determinants are frequently found in multiresistance plasmids, such as pSM39 [57]. This is an example of how the co-localization of resistance to heavy-metals, biocides and antibiotics may provide a selective advantage, due to co-selection and consequent co-resistance, of strains carrying these different elements.

Another example of the importance of co-resistance and co-selection between antimicrobial agents is the linkage between *qacA* and the β-lactamase gene *blaZ* that occurs in pSM39, and has also been reported on multiresistance plasmids of staphylococci isolated from both animal and humans [4, 58]. The *qacA* gene can also be encountered in plasmids together with genes encoding resistance to trimethoprim, aminoglycosides and fosfomycin [24]. Recently, a report by Lee *et al*., showed a high correlation between carriage of *qacA/B* and *smr* genes in mupirocin-reistant MRSA in Korean hospitals, including high-level resistance due to the plasmid-encoded *mupA* gene [31]. A debatable example of co-selection is the possible selective advantage of MRSA strains attributable to the presence of biocide resistance genes. Although some studies have found no linkage between MRSA and *qacA/B* carriage [30], others studies suggest the opposite [2, 39]. Particularly, a recent study by Zhang *et al*. has showed that *qac* genes were more frequent in MRSA strains that colonized nurses than the general public [71]. More recently, a study has shown how the introduction of a program for MRSA decolonization based on the use of chlorhexidine, although resulting in an overall reduction of the incidence of MRSA infections, also promoted the selection of one specific MRSA clone, which showed a higher prevalence of *qacA* carriage [45].

A surprising finding of the sequence analysis of plasmids pSM39 and pSM52 was the evidence that both could result from rearrangements between plasmids originating in *S. aureus* and coagulase-negative staphylococci. In the particular case of pSM39 this rearrangement occurred most probably with a plasmid from *S. epidermidis*. Interestingly, plasmid pSM52 was partially identical to plasmid pSK108 while pSM39 shared a common region with plasmid pSK105. These two plasmids, pSK108 and pSK105, were originally identified on the same strain of *S. epidermidis* [32]. Transfer of

plasmids and other MGEs between *S. aureus* strains of diverse origin (e.g., human, animal) as well as between different staphylococcal species have been reported [34, 36, 57]. This finding has also been reported for plasmids carrying the biocide resistance genes *qacA* and *smr* [32, 33]. Our data strengthens the importance of an endeavor to portray the distribution of plasmids between staphylococci, not only from human (clinical), but also from animal and environmental origin.

Efflux-mediated resistance to fluoroquinolones in *S. aureus*

Fluoroquinolone resistance in *S. aureus* emerged and propagated rapidly after the introduction of these antibiotics in clinical practice [21]. Nowadays, in Europe, fluoroquinolone-resistant isolates account for up to 25% of the *S. aureus* clinical isolates, a proportion rate that increases up to 90% among MRSA isolates [17]. This increased rate has been explained by clonal spread of fluoroquinolone-resistant MRSA strains and selection by fluoroquinolone use [21, 48, 70]. Studies have been presented that support the co-selection of MRSA strains by fluoroquinolone pressure. A study by Charbonneau *et al.*, reported that the reduction of fluoroquinolones prescription in a French hospital was followed by a reduction in the rates of MRSA [7]. A subsequent study in the same hospital, conducted by Parienti and colleagues, showed that, after reintroduction of fluoroquinolones, the MRSA rates increased again [46]. Also, a recent study by Holden *et al.*, demonstrated that the pandemic spread of the clone EMRSA-15 is closely related with the emergence of resistance to fluoroquinolones [20]. Despite the rare use of fluoroquinolones to treat staphylococcal infections, the wide use of these antibiotics in the hospital setting has been a major driving force in the maintenance and dissemination of *S. aureus* resistant strains.

In the work carried out to explore the contribution of efflux on resistance to fluoroquinolones (Chapters 5 to 7), the application of the EtBr-agar Cartwheel method in the collection of 52 ciprofloxacin-resistant clinical isolates revealed a high ability of differentiation of the isolates according to their efflux capacity, allowing the definition of three distinct groups of isolates. Fluorometry assays to evaluate directly the efflux activity present in the isolates and determination of MICs in the presence of efflux

inhibitors corroborated the previous distinction of the isolates as efflux-negative (EtBrCW-negative), efflux-intermediate and efflux-positive (EtBrCW-positive). A significant proportion of isolates, 37%, presented increased efflux activity. In comparison, in a study by DeMarco *et al.*, a proportion of 49% of strains with increasing activity was found among a collection of 232 bloodstream *S. aureus* isolates [14]. The increased efflux activity found for the efflux-positive isolates was correlated with increased resistance to fluoroquinolones (Chapters 5 and 6). It was demonstrated that efflux is an important component of fluoroquinolone resistance in those isolates. It could be ascertained that, independently of the target mutations in GrlA and GyrA proteins carried by the isolates, these accounted for levels of resistance of 8 to 32 mg/L for ciprofloxacin and 32 to 128 mg/L for norfloxacin. The remaining level of resistance, up to 256 mg/L for ciprofloxacin and 1024 mg/L for norfloxacin could be attributed to efflux (Chapters 5 and 6). The addition of known efflux inhibitors resulted in a reduction of the resistance levels but did not render the isolates susceptible to fluoroquinolones (Chapter 5), with the exception of a single isolate, SM15 (Chapter 6). This strain showed an intermediate efflux activity and carried a single mutation in GrlA (E84Q), representing an intermediate stage of the development process of fluoroquinolone resistance, where the contributions of mutation and efflux to this resistance are proportional. We were able to demonstrate that efflux was determinant for the resistance phenotype presented by SM15, as the addition of thioridazine reduced the MICs of ciprofloxacin and norfloxacin from 8 mg/L and 16 mg/L, respectively, to 1 mg/L and 4 mg/L, respectively. These values correspond to susceptibility according to the guidelines established by CLSI [9] and EUCAST [60], although the latter does not have breakpoint values defined for norfloxacin. Thus, a reversion of the fluoroquinolone resistance phenotype was achieved in SM15. These results are of particular relevance, since they indicate that efflux activity may develop an important role in an early stage of acquisition of mutations in the fluoroquinolone target genes.

The hypothesis that efflux is a major player in the development of fluoroquinolone resistance was further scrutinized in Chapter 7. In this Chapter we presented the work carried out on the prolonged exposure of three *S. aureus* strains, the susceptible reference strain ATCC25923 and two ciprofloxacin-resistant clinical strains SM50 and SM2, to constant concentrations of ciprofloxacin, ethidium bromide and

cetrimide. The exposure processes performed yielded strains with a multidrug resistance phenotype, independently of the efflux inducer used; all strains presented an increased resistance level to fluoroquinolones for the two clinical strains and the development of a fluoroquinolone resistance phenotype for ATCC25923 in addition to a reduced susceptibility to biocides and dyes. We showed that for the two clinical strains, the registered increase in resistance levels of ciprofloxacin from 32-64 mg/L to 256-512 mg/L and of norfloxacin from 64-128 mg/L to >512 mg/L was solely attributable to an increase in efflux activity in these strains, as no additional fluoroquinolone-resistance mutations were detected in the target genes. In the particular case of ATCC25923, this strain presented at the end of any of the exposure processes, a MIC of ciprofloxacin of 2 mg/L and for norfloxacin, it showed MIC values of 8 mg/L or higher, values that correspond to intermediate resistance or resistance, according to the CLSI and EUCAST guidelines. These resistance levels were due solely to an increase in efflux activity in all the exposure processes, with the exception of the exposure to the inhibitory concentration of ciprofloxacin. In this case, efflux was the first response of the cell to the stress imposed by the fluoroquinolone, as indicated by the overexpression of efflux pump genes during the first day of exposure that was later on overcome by the acquisition of a mutation in GrlA, namely S80F. So, by exposing a fully susceptible strain to an inhibitory concentration of ciprofloxacin, we were able to demonstrate one of our main premises, i.e., that development of fluoroquinolone resistance is a step-wise process, beginning with a first efflux-mediated response that was complemented with the gain of a mutation associated with fluoroquinolone resistance.

Efflux-mediated resistance to biocides in *S. aureus*

As mentioned previously, the clinical relevance of reduced susceptibility to antiseptics and disinfectants in clinical isolates is a matter of debate. This resistance has been mainly correlated to plasmid-encoded efflux pumps. The role of MDR efflux pumps encoded in the chromosome has been met with little interest, despite the fact that most of them have the ability to transport these compounds.

In Chapter 6, we explored the possible efflux-mediated cross-resistance between fluoroquinolones and biocides in the 52 ciprofloxacin-resistant isolates. Assessing the MICs of biocides and dyes in the three groups of strains showing different degrees of efflux activity revealed that the efflux-positive and -intermediate isolates presented higher MICs of biocides and dyes. This reduced susceptibility profile was more evident for the dye EtBr; for the QACs cetylpyridinium chloride, benzalkonium chloride, cetrimide, dequalinium chloride and tetraphenylphosphonium bomide; and for the biguanidine chlorhexidine digluconate. These results allowed us to establish a link between reduced susceptibility to fluoroquinolones and to biocides in those isolates with increased efflux activity. We reinforced this finding in Chapter 7, by demonstrating that prolonged exposure of three *S. aureus* strains to a fluoroquinolone, a biocide or a dye resulted in strains with cross-resistance to those agents.

The increased levels of MICs of biocides found in the collection of isolates (Chapter 6) and in the exposed strains (Chapter 7) were two to eight-fold higher than the ones of efflux-negative isolates or unexposed strains, indicating that the bacteria can endure higher concentrations of these compounds and may be more fit to survive in environments where these compounds are present, such as in healthcare settings. Current protocols in several hospitals for MRSA decolonization of colonized patients rely on the use of antiseptics, such as chlorhexidine, benzalkonium chloride and cetrimide, among others. In the UK, a nationwide program was delineated with defined guidelines for skin decolonization that comprise the use of washing solutions of chlorhexidine, povidone-iodine, triclosan or benzalkonium chloride in combination with hair wash with an antiseptic detergent [10]. Similar guidelines can be found for other countries, including Portugal, in which the use of antiseptics is recommended for skin decolonization and disinfection prior to invasive procedures. In addition, hand hygiene of patients and healthcare workers is a mainstay of nosocomial infection control and prevention. Hand formulations consist mainly of alcohol solutions, many times supplemented with QACs, or on chlorhexidine solutions. Besides, disinfectant agents are recommended for disinfection of medical equipment as well as for the cleansing and disinfection of surfaces [55]. Consequently, the hospital is a favorable environment for the emergence and maintenance of clinical strains presenting increased resistance to biocides. Even though the in-use concentrations for these compounds is much higher

than the increased MIC levels registered here (as an example, chlorhexidine is usually used in formulations at up to 4% and the increased MICs registered in our isolates and exposed strains goes up to 0.000125%), a misuse of these formulations could lead to environments with residual concentrations of biocides that could promote the persistence of strains with reduced susceptibility to these compounds. Although a matter of debate, some authors state that one can not overlook the presence of biocide residues in the environment that together with the misuse of biocide formulations (application of inadequate concentrations and/or inappropriate period of times, incorrect use of the product, the "topping-up" use of biocide formulations) could create environments where bacteria are exposed to sub-lethal concentrations and thus potentiate the emergence of reduced susceptibility to these compounds [35, 40 53, 54]. Our own results on the exposure to sub-inhibitory concentrations of cetrimide (Chapter 7) show that the prolonged contact with this agent could promote an efflux-mediated multidrug resistance phenotype to the *S. aureus* cells. In the already mentioned study by Furi *et al.*, another finding was the link between increased MICs of benzalkonium chloride and chlorhexidine by two-fold increase and cross-resistance to fluoroquinolones in clinical isolates and mutants with alterations in the *norA* promoter region (and probable *norA* increased transcription) [19]. In addition, a study by Huet *et al.*, showed that exposure to sub-lethal concentrations of several biocides promoted an efflux-mediated response by *S. aureus*, resulting in a progeny with increased resistance to those agents [23].

Assessing the individual contribution of chromosomally-encoded multidrug efflux pumps to efflux-mediated resistance: a major challenge

One of the main aims of this Thesis was to ascertain the individual contribution of multidrug efflux pumps to the resistance phenotypes. This task was found to be successful when evaluating plasmid-encoded efflux pumps, since the generation of defined genetic systems is simple to achieve, therefore allowing to single out the contribution of those systems to efflux-mediated resistance in *S. aureus*. Focusing this analysis on chromosomally-encoded efflux pumps proved to be not a straightforward

approach, as already discussed above. The interplay between the main chromosomal multidrug efflux pumps was explored in Chapters 5 and 7. The main observations taken from Chapter 5, where a sub-set of clinical isolates (with basal and increased efflux activity) and the reference strain ATCC25923 were subjected to a single exposure to a sub-inhibitory concentration of ciprofloxacin or ethidium bromide, were the following: (i) only low-levels of efflux pump gene overexpression were found in the sub-set of clinical isolates; (ii) no significant difference was observed between the two groups of isolates with differing efflux activities and no pattern of expressed genes regarding the efflux inducer or efflux activity of the isolates was defined; (iii) the response of the clinical isolates to the inducer agents differed significantly from the response of the reference strain, which showed higher levels of overexpression for all efflux pump genes tested; (iv) increasing the concentration of the inducer promoted higher levels of gene expression and/or the expression of additional genes. Overall, these results indicated that *S. aureus* clinical isolates may be primed for efflux due to their previous exposure to antimicrobials in the hospital environment, and that the efflux-mediated response to stress is dependent of the concentration of the stimulus.

The strategy used in Chapter 7 was distinct; here we submitted strains differing in their efflux capacity and susceptibility to fluoroquinolones to a constant presence of two concentrations of different inducers (ciprofloxacin, cetrimide and ethidium bromide), over a twenty-day period. The main findings of this study were: (i) exposure to efflux inducers promoted an efflux-mediated response that varied over time, generally an early-response with high levels of expression of several efflux pump genes followed by a late-response, characterized by overexpression of specific genes; (ii) the overall cell response to the efflux inducers varied according to the strains original efflux capacity, being more pronounced for the strains with a low initial efflux activity; (iii) some degree of specificity was observed in the genes overexpressed in response to ciprofloxacin (mainly *norA*, *norB* and *norC* genes), and to ethidium bromide (the *mepA* gene).

In sum, we have found that *S. aureus* can cope with stress by a multiplicity of patterns of efflux-mediated responses. This contrasts with what is normally found for Gram-negative bacteria such as *E. coli*, where the efflux system AcrAB-TolC is a preferential response by the cell. In our work, we have found that *S. aureus* strains use

all the available efflux systems to respond to noxious compounds, without a single pump to be preferentially recruited and that even the same strain can show different responses to the same agent, depending on the exposure conditions. One of the best examples of this versatility of responses was provided by ATCC25923, which under different conditions of stress, namely, inducer agent, its concentration and time of contact with the inducer agent responded in different ways. For this strain, single exposure to a sub-inhibitory concentration of ethidium bromide originated overexpression of all efflux pump genes tested. The same strain exposed to the same agent but over a prolonged period of time and to increasing inhibitory concentrations showed a specific NorA-mediated response. Finally, when this same strain was exposed to a constant inhibitory concentration of ethidium bromide, it showed a first, non-specific response with all efflux pump genes available, after which it shifted to the overexpression of a single gene, *mepA*. In literature, the scarce studies conducted on efflux-mediated resistance in *S. aureus*, either with isolates of distinct origins (clinical, environmental) and from diverse geographical points, or with laboratory-derived strains/mutants, also supports the use by *S. aureus* cells of a myriad of efflux-mediated responses [14, 23, 27, 28]. However, in a recent study by Kwak *et al.* with clinical isolates from Korea, the authors were able to correlate overexpression of the *norB* gene with higher ciprofloxacin resistance in MRSA isolates, a link that could not be established with MSSA isolates [29]. Nevertheless, in all these studies the most common genes found overexpressed and associated with resistance phenotypes are the *mepA* and the *nor* genes, indicating that in the future, work should be focused on these efflux systems, inasmuch as recent studies have unraveled new potential roles for some of these pumps [15, 16, 64, 65].

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CHAPTER 9

Conclusions and Future Perspectives

The work performed under the scope of this Thesis aimed to assist on the elucidation of the mechanisms that trigger efflux-mediated resistance in *S. aureus* and the contribution of this mechanism to resistance towards different antimicrobials in clinical isolates. It also opened several research avenues that remain to be explored in future work.

Part I of this Thesis was dedicated to the characterization of some of the main *S. aureus* multidrug efflux pumps, namely the chromosomally-encoded NorA and the plasmid-encoded QacA and Smr. Concerning NorA, we were able to demonstrate the contribution of this pump to reduced susceptibility to fluoroquinolones, biocides and dyes and its role in the response to prolonged stress in a reference, pan-susceptible strain. We also showed that the regulation of this pump is complex, with several factors involved in the modulation of the expression of the *norA* gene and on the activity of NorA. We also evaluated the role of this pump to the resistance phenotypes on a representative sub-set of a collection of 52 ciprofloxacin resistant *S. aureus* clinical isolates. Two circulating *norA* alleles, and their variants, were found among this sub-set of isolates, yet, their association to resistance phenotypes towards different antimicrobials was difficult to establish. The overall information gathered in Chapter 2 highlights the need to perform more studies on this important efflux system, namely the determination of the effect of mutations in NorA activity, particularly Gly-147→ Ser and Phe-303→ Tyr, as well as the role of alterations in the *norA* promoter region in the expression levels of the gene. The studies on *norA* regulation also remained with some issues in need of clarification, namely the actual impact of the regulator MgrA on the modulation of *norA* expression. We were able to establish an association between MgrA and *norA* overexpression, but further studies are required to ascertain the respective mechanism, including the determination of the cellular ratio of the phosphorylated and non-phosphorylated MgrA in the strains studied that should provide further insights on the impact of MgrA upon *norA* expression. Nevertheless, we were able to demonstrate that increased *norA* expression in the EtBr-adapted strain was attributable to an increase in the transcription rate instead of higher stability of the *norA* mRNA.

The studies on the plasmid-encoded efflux pumps QacA and Smr allowed us to correlate their activity with reduced susceptibility to biocides and dyes in the clinical isolates that carried these efflux pump determinants. QacA was found to be associated

with resistance to a wider panel of antimicrobial compounds and to higher levels of resistance than Smr, namely several quaternary ammonium compounds, such as benzalkonium chloride and dequalinium chloride, diamidines and the biguanidine chlorhexidine. They also differed in their genetic environment, while *qacA* gene was found in a large non-conjugative multiresistance plasmid, pSM39; *smr* was carried by a small non-conjugative plasmid, pSM52, which carried no additional resistance determinants. Full sequence determination of plasmids pSM39 and pSM52 suggested that they could have resulted from rearrangement between plasmids originating in *S. aureus* and other staphylococci strains. These findings reveal the relevance of plasmids such as the ones here described in providing *S. aureus* with cross-resistance to multiple antimicrobial agents and as a selective advantage for persistence in distinct environments. The distribution and detailed analysis of plasmids from other staphylococcal species is an area still relatively unexplored, and deeper insights into the dynamics of plasmid mobility between staphylococci, including from different sources (clinical, animal, environmental) would surely provide important knowledge on the dissemination and acquisition of efflux determinants and their relevance for antibiotic resistance.

Part II of this Thesis was dedicated to the evaluation of the contribution of efflux to resistance to fluoroquinolones and biocides in a collection of 52 ciprofloxacin-resistant *S. aureus* clinical isolates. Data gathered in Chapters 5 and 6 allowed us to correlate increased efflux activity in those isolates with increased resistance to those antimicrobial agents.

Reduced susceptibility to several quaternary ammonium compounds, including benzalkonium chloride, cetrimide and cetylpyridinium chloride, and in a lesser extent to dequalinium chloride and to the biguanidine chlorhexidine, was associated with increased efflux activity by determination of MICs in the absence and presence of efflux inhibitors. However, this approach may not fully account for the effects of biocides on *S. aureus* cells. Thus, continuing this work with the use of methodology that assays the lethality effects of biocides upon the cells and the factors that affect their efficacy, both *in vitro* and in-use conditions, such as testing the rate-of-kill of bacteria by biocides, would complement these studies and provide information on whether the increased

efflux activity can be correlated with reduced lethality by biocides and ensuing higher survival of these strains.

In the particular case of fluoroquinolones, the results obtained through this Thesis demonstrated that efflux is an important component of resistance to these antibiotics, together with target-based mutations. Furthermore, it indicated that efflux mediates a first-line response to fluoroquinolones. This hypothesis was further explored in Part III of this Thesis. We were able to show that exposure of a susceptible strain to ciprofloxacin could promote the development of a resistance phenotype in a two-step process, a first response with augmented efflux followed by the occurrence of target-based mutations that would then confer the cell a stable resistance phenotype. This result is a major finding of our work, as it demonstrates that efflux is a primary mechanism of the *S. aureus* cell to cope with stress and may have important implication in the management of this pathogen.

One of the focus of our research group is the evaluation of the efficacy of efflux inhibitors in the reduction/reversal of resistance and their potential use as co-adjuvants in antimicrobial therapy. One of the main findings of this Thesis is the premise that efflux is a first-line response to antimicrobial agents, particularly fluoroquinolones, hinting at the potential role of efflux inhibitors in prevention of emergence of resistance in *S. aureus*. Further efforts could be undertaken to ascertain if, in fact, efficient efflux inhibitors have the ability to prevent/delay the emergence of fluoroquinolone resistance in *S. aureus.*

Throughout the studies conducted we have been able to show that *S. aureus* can present a multiplicity of complex efflux-mediated responses that vary with the pressure that the cells are exposed to. We are still in the beginning stages of comprehending how these diverse responses to stress are triggered and the interplay between the several bacterial efflux pumps. One of the main issues that remains to be fully clarified is the assessment of the basal levels of expression in clinical strains, as well as the correlation between the levels of efflux pump gene expression and the actual quantification of efflux proteins in the cell membrane and their activity. The lack of appropriate experimental tools to further explore these questions is one of the main obstacles in this area of work and will probably involve much work and effort in the years to come.

In conclusion, the work presented in this Thesis demonstrated that efflux is an important component of resistance to fluoroquinolones and biocides in the major pathogen *S. aureus* and may be a key player in the development of resistance as a first-line response to antimicrobials. Many questions are still in need of clarification, justifying further studies on this still relatively unknown mechanism, yet pivotal for the survival of the ever-evolving and ever-challenging *S. aureus*.

Annexes

Annex A – Supplementary material for Chapter 6, Resistance to antimicrobial compounds mediated by efflux.

Table S1. MIC values of ciprofloxacin and norfloxacin for strains representative of the EtBrCW-positive, EtBrCW-intermediate and EtBrCW-negative groups, in the absence and presence of subinhibitory concentrations of the efflux inhibitors thioridazine and verapamil.

Strain	MIC (mg/L)					
	CIP			NOR		
	No EI	+ TZ	+ VER	No EI	+ TZ	+ VER
EtBrCW-positive						
SM14	256	32	128	1024	128	256
SM50	64	16	16	256	32	64
SM52	16	8	8	64	32	64
EtBrCW-intermediate						
SM15	8	1	4	16	4	4
SM22	128	16	64	512	128	256
SM31	64	32	64	256	128	128
SM44	256	32	128	512	64	128
EtBrCW-negative						
SM2	32	16	16	128	32	64
SM3	16	8	8	64	32	64
SM4	8	8	8	64	32	64

CIP: ciprofloxacin; NOR: norfloxacin; EI: efflux inhibitor; TZ: thioridazine; VER: verapamil. Thioridazine and verapamil were used at 12.5 mg/L and 200 mg/L, respectively.

Table S2. MIC values of ethidium bromide for strains representative of the EtBrCW-positive, EtBrCW-intermediate and EtBrCW-negative groups, in the absence and presence of subinhibitory concentrations of the efflux inhibitors thioridazine and verapamil.

Strain	EtBr MIC (mg/L)		
	No EI	+ TZ	+ VER
EtBrCW-positive			
SM14	16	4	4
SM50	8	1	2
SM52	16	1	4
EtBrCW-intermediate			
SM22	16	4	8
SM31	16	2	4
SM44	16	2	4
EtBrCW-negative			
SM2	8	2	2
SM3	2	1	1
SM4	4	2	2

EtBr: ethidium bromide; EI: efflux inhibitor; TZ: thioridazine; VER: verapamil. Thioridazine and verapamil were used at 12.5 mg/L and 200 mg/L, respectively

Table S3. MIC values of biocides for strains representative of the EtBrCW-positive, EtBrCW-intermediate and EtBrCW-negative groups, in the absence and presence of subinhibitory concentrations of the efflux inhibitors thioridazine and verapamil.

Strain	MIC (mg/L)															MIC (%)		
	CET			CPC			BAC			TPP			DQ			CHXg		
	No EI	+ TZ	+ VER	No EI	+ TZ	+ VER	No EI	+ TZ	+ VER	No EI	+ TZ	+ VER	No EI	+ TZ	+ VER	No EI	+ TZ	+ VER
EtBrCW-positive																		
SM14	8	4	4	4	1	1	4	2	2	64	16	16	16	8	8	0.000125	0.00006	0.00006
SM50	4	0.5	2	1	0.125	0.5	2	0.25	1	32	8	16	4	2	4	0.00006	0.000015	0.00003
SM52	8	2	4	2	0.03	1	2	0.25	2	16	1	8	4	2	4	0.00006	0.000015	0.00003
EtBrCW-intermediate																		
SM22	8	4	8	2	0.5	1	4	1	2	64	8	32	16	8	16	0.000125	0.00006	0.00006
SM31	8	2	4	2	0.25	0.5	2	0.5	1	32	8	16	8	4	8	0.000125	0.00003	0.00006
SM44	8	4	8	2	0.25	1	4	1	2	32	8	16	8	4	8	0.000125	0.00003	0.00006
EtBrCW-negative																		
SM2	2	0.5	1	0.5	0.03	0.25	1	0.125	0.25	32	4	8	4	2	2	0.00006	0.000015	0.00003
SM3	2	0.25	0.5	0.5	0.03	0.5	1	0.125	0.5	16	2	8	2	2	2	0.00003	0.000015	0.000015
SM4	2	0.5	1	0.5	0.06	0.25	1	0.25	0.25	16	4	8	4	4	2	0.00003	0.000015	0.000015

CET: cetrimide; CPC: cetylpyridinium chloride; BAC: benzalkonium chloride; TPP: tetraphenylphosphonium bromide; DQ: dequalinium chloride; CHXg: chlorhexidine digluconate; EI: efflux inhibitor; TZ: thioridazine; VER: verapamil. Thioridazine and verapamil were used at 12.5 mg/L and 200 mg/L, respectively.

Annex B – Supplementary material for Chapter 7, Efflux as a first-line response to antimicrobial in *S. aureus*.

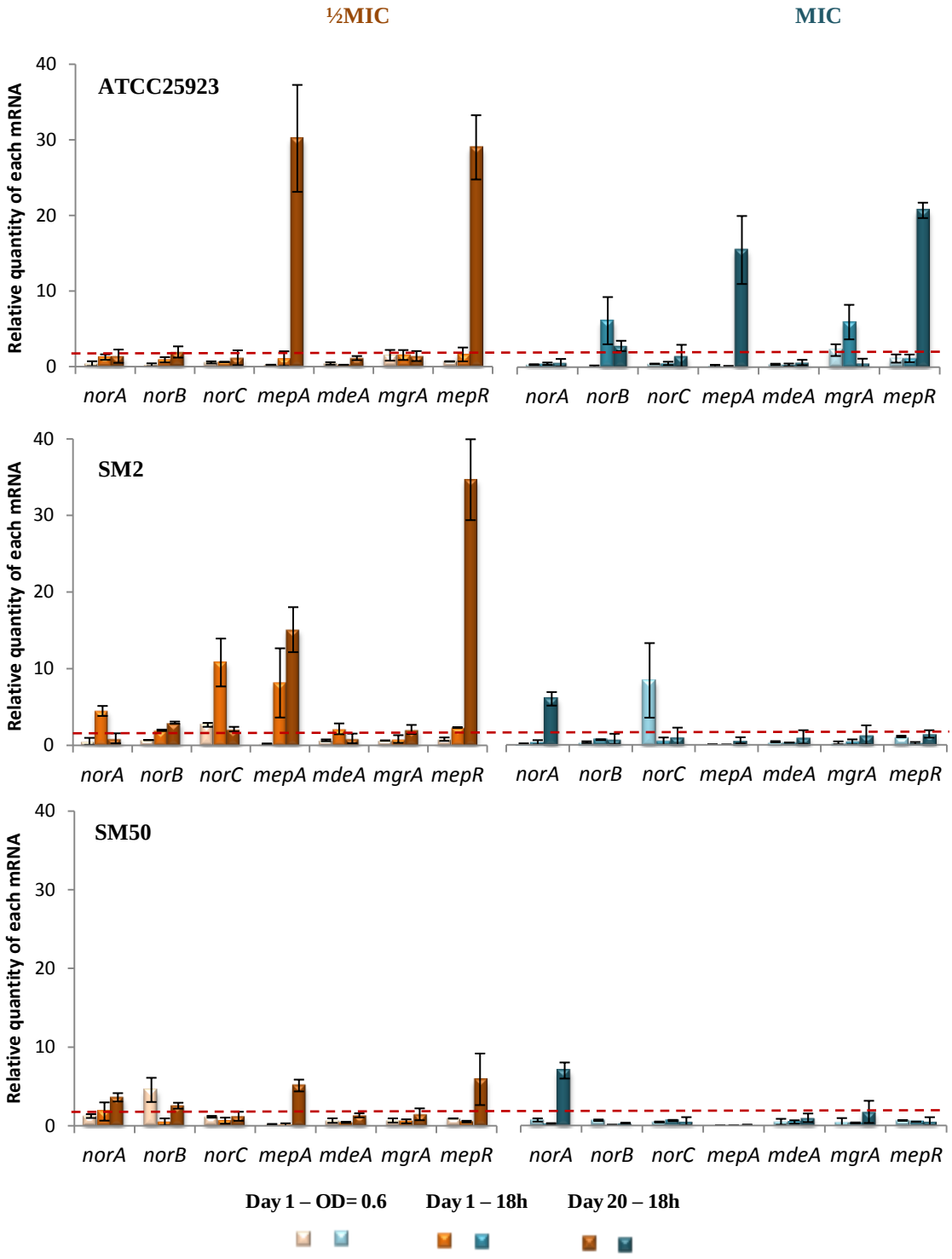

Figure S1. Quantification of the expression of efflux pump genes and regulators by RT-qPCR of strains ATCC25923, SM2 and SM50 at different time points of the exposure to EtBr at ½ MIC (orange) and to EtBr at MIC (blue). Gene expression was measured in the presence of EtBr relatively to the inducer-free condition. The results are presented as the mean and standard deviation of at least two independent assays performed with extracted total RNA. Overexpression was considered for values superior to 2 (cut-off value represented by the red line).

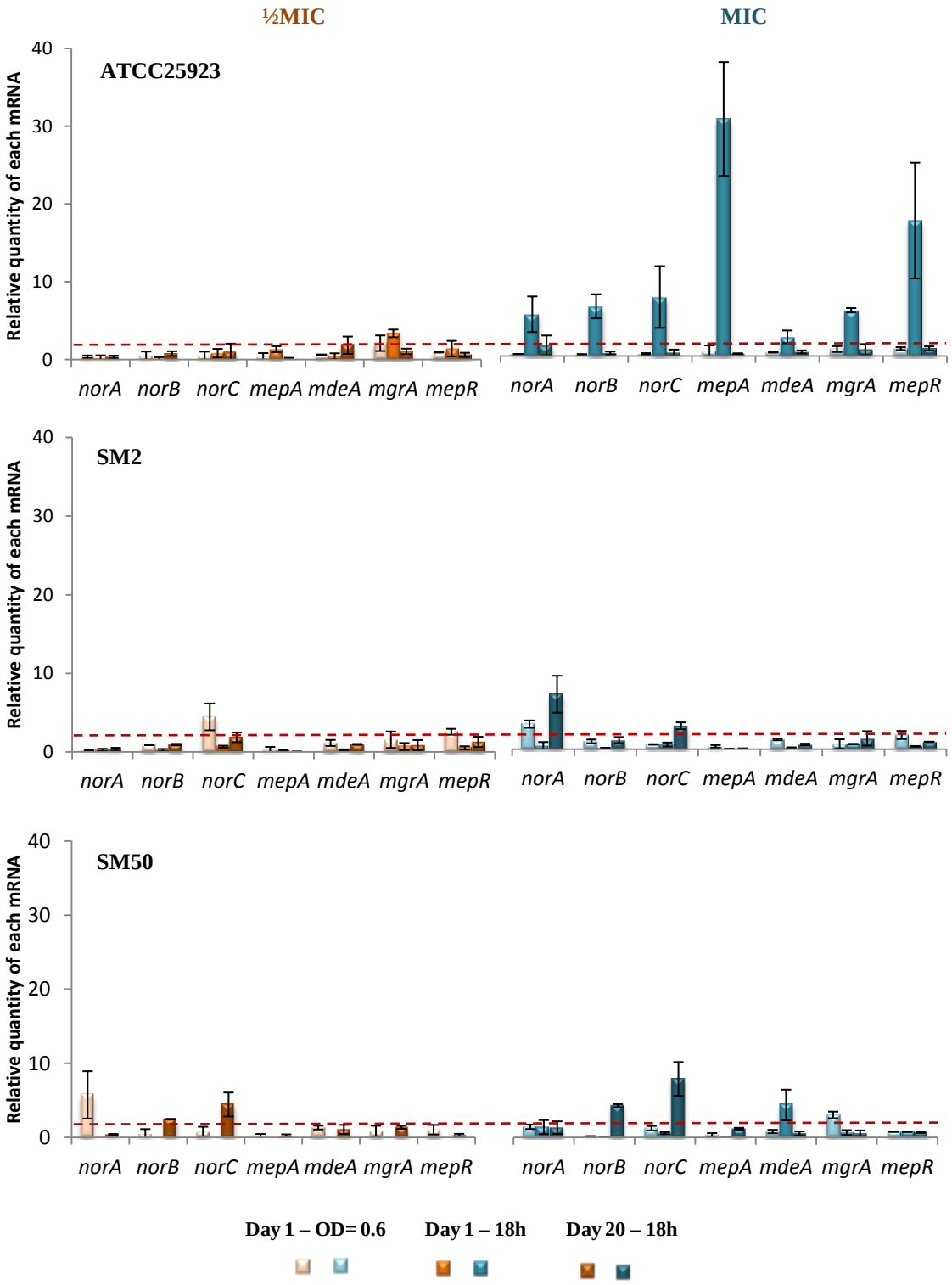

Figure S2. Quantification of the expression of efflux pump genes and regulators by RT-qPCR of strains ATCC25923, SM2 and SM50 at different time points of the exposure to CIP at ½ MIC (orange) and to CIP at MIC (blue). Gene expression was measured in the presence of CIP relatively to the inducer-free condition. The results are presented as the mean and standard deviation of at least two independent assays performed with extracted total RNA. Overexpression was considered for values superior to 2 (cut-off value represented by the red line).

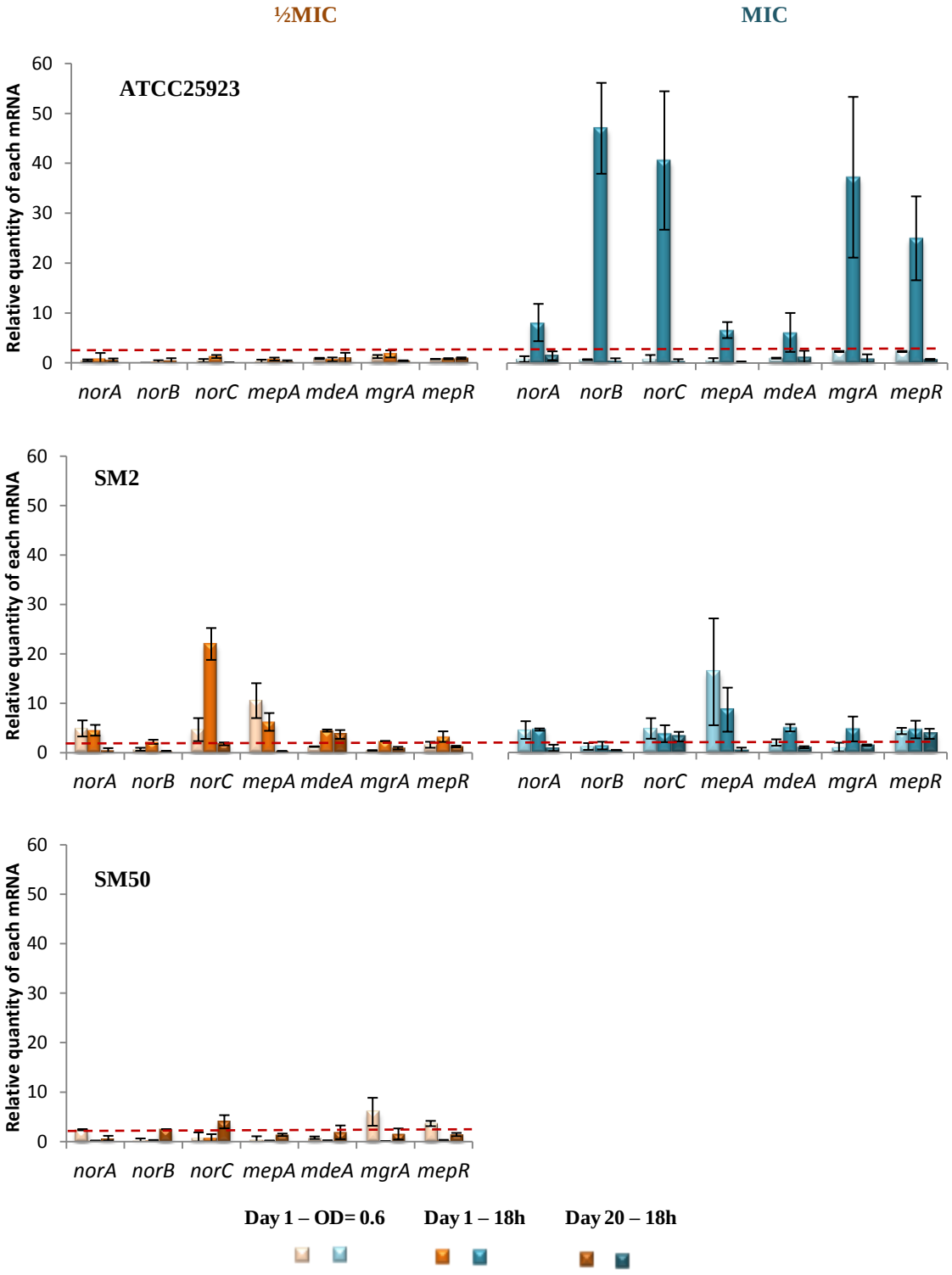

Figure S3. Quantification of the expression of efflux pump genes and regulators by RT-qPCR of strains ATCC25923, SM2 and SM50 at different time points of the exposure to CET at ½ MIC (orange) and to CET at MIC (blue). Gene expression was measured in the presence of CET relatively to the inducer-free condition. The results are presented as the mean and standard deviation of at least two independent assays performed with extracted total RNA. Overexpression was considered for values superior to 2 (cut-off value represented by the red line).